Remarks

Applicants have herein amended claims 11, 12, 32, 33, 48, 49, 70, and 71, in compliance with requests by the Examiner (discussed further below). Applicants have also amended claims 38 and 61 to specify that "the polypeptide encoded by the HEMCM42 cDNA is at least 95% identical to SEQ ID NO:59". And, Applicants submit herewith a Supplemental Application Datasheet (ADS) to amend the inventive entity to coincide with the currently claimed subject matter. Additionally, to comply with PTO guidelines the Domestic Priority Information section of the Supplemental ADS has also been amended to use the phrase "An application claiming the benefit under 35 USC 119(e)" in place of the former phrase "Non-provisional of".

Claims 16-23, 37, 53-60 and 75 have been canceled herein without prejudice or disclaimer. Applicants reserve the right to pursue the subject matter encompassed by all canceled claims in one or more divisional or continuation applications.

Support for the amendments to claims 38 and 61 can be found throughout the specification as filed. In particular, support for amended claims 38 and 61 can be found at, for example, page 73, line 32 to page 74, line 20 and page 100, line 27 to page 101, line 5 (95% identity). Thus, no new matter has been added.

Claims 1-10, 13, 14, 24-31, 34, and 35 have been allowed. Claims 1-15, 24-36, 38-52 and 61-74 are currently pending.

Formal Matters

A. Amendment of the Specification

In compliance with the Examiner's request (*see* Paper No. 08232004, page 2, paragraph no. 2), Applicants have herein amended the first paragraph (cross-reference to related applications) to indicate the status of related applications. Applicants have also amended the first paragraph to bring it into compliance with recent PTO guidelines.

B. Amendment of Inventorship

Applicants respectfully request consideration and entry of the present amendment to correct inventorship pursuant to 37 C.F.R. §1.48(b) (*Non-provisional application - fewer inventors due to amendment or cancellation of claims*). The presently pending claims are directed to HEMCM42 antibody embodiments. In this regard, the undersigned has been informed that the inventive entity of the subject matter encompassed by the claims is: Steven M. Ruben, Craig A. Rosen, and Gregory A. Endress. Accordingly,

Applicants request that the presently pending application be amended to show the above three persons as inventors. Thus, please remove the following names from the list of inventors: Kenneth C. Carter, Patrick J. Dillon, Guo-Liang Yu, Jian Ni, and Ping Feng.

Rejections under 35 U.S.C. § 112, Second Paragraph

A. Claims 11, 12, 22, 23, 32, 33, 48, 49, 59, 60, 70, and 71

Claims 11, 12, 22, 23, 32, 33, 48, 49, 59, 60, 70, and 71 were rejected under 35 U.S.C. §112, second paragraph. In particular, it was alleged that the claims at issue lack appropriate antecedent basis. *See*, Paper No. 08232004, page 3, paragraph no. 5(A).

As an initial matter, claims 22, 23, 59, and 60 have been cancelled herein. Therefore, the rejection of these claims under 35 U.S.C. §112, second paragraph has been rendered moot.

Additionally, Applicants have herein amended claims 11, 12, 32, 33, 48, 49, 70, and 71, according to suggestions provided by the Examiner. *See*, Paper No. 08232004, page 3, paragraph no. 5(A). Therefore, Applicants respectfully that the rejection of pending claims 11, 12, 32, 33, 48, 49, 70, and 71 under 35 U.S.C. §112, second paragraph, be reconsidered and withdrawn.

B. Claims 22, 23, 59, and 60

Claims 22, 23, 59, and 60 were rejected under 35 U.S.C. §112, second paragraph. *See,* Paper No. 08232004, page 4, paragraph no. 5(B).

Without acquiescing to the instant rejection, Applicants have herein canceled claims 22, 23, 59, and 60 without prejudice or disclaimer, thereby rendering this rejection moot. Applicants reserve the right to pursue claims drawn to all canceled subject matter in one or more divisional or continuation applications.

Rejections under 35 U.S.C. § 112, First Paragraph

A. Claims 17-23

Claims 17-23 were rejected under 35 U.S.C. §112, first paragraph. *See*, Paper No. 08232004, page 5, paragraph no. 7. Without acquiescing to the instant rejection, Applicants have herein canceled claims 17-23 without prejudice or disclaimer, thereby rendering this rejection moot. Applicants reserve the right to pursue claims drawn to all canceled subject matter in one or more divisional or continuation applications.

B. Claims 38-75

Claims 38-75 were rejected under 35 U.S.C § 112, first paragraph, because although the specification is "enabling for an antibody that binds a protein encoded by cDNA HEMCM42 that consists of SEQ ID NO: 13 and that encodes the polypeptide consisting of SEQ ID NO:59," the specification allegedly "does not reasonably provide enablement for an antibody that binds any polypeptide encoded by the cDNA HEMCM42." *See*, Paper No. 08232004, page 7, paragraph no. 8.

As an initial matter, Applicants note that there appears to be a clerical error in the presently pending Office Action because SEQ ID NO:13 was referenced as being associated with the presently pending claims (*i.e.*, as the translated nucleotide sequence for the cDNA HEMCM42). Applicants note that as shown in Table 1, row 3, column 4 on page 64, the corresponding nucleotide sequence for the cDNA HEMCM42 is SEQ ID NO:23. Thus, Applicants will respond to the rejection as it pertains to the cDNA HEMCM42, SEQ ID NO:23, and the encoded polypeptide SEQ ID NO:59.

Applicants respectfully disagree, and assert that the previously pending claims were fully enabled as filed. However, notwithstanding the above, Applicants have canceled claims 54-60 and have amended claims 38 and 61 (from which claims 39-53 and 62-75 depend, respectively) to recite "wherein said polypeptide encoded by the HEMCM2 cDNA is at least 95% identical to SEQ ID NO:59." Accordingly, in view of the cancellation of claims 54-60 and the amendments to claims 38 and 61, Applicants request that the rejection of claims 38-75 under 35 U.S.C. § 112, first paragraph be reconsidered and withdrawn.

To the extent that the instant rejection may be applied against the claims as amended, Applicants respectfully traverse.

In the present Office Action it was alleged that "it is not clear if cDNA HEMCM42 is limited to consisting of SEQ ID NO: 13" and "[t]herefore, the claims have been interpreted in the broadest reasonable manner in light of the specification to include **any** protein encoded by the cDNA HEMCM42." *Id.* at page 7, second full paragraph (emphasis in original). In response, Applicants emphasize that the amendments made herein limit claims 38-53 and 61-75 to a specific genus of fragments and variants of the disclosed HEMCM42 polypeptides which share at least 95% identity to SEQ ID NO:59. (Support for amended independent claims 38 and 61 can be found at, for example, page 73, line 32 to page 74, line 20 and page 100, line 27 to page 101, line 5).

Moreover, the specification teaches that the polypeptide encoded by the HEMCM42 clone contained within ATCC Deposit Number 209075 and the polypeptide of SEQ ID NO: 59, are meant to encompass the same polypeptides of Gene No. 13 as disclosed in Table 1, on page 64 of the specification. Specifically, the specification teaches that:

> [t]he nucleotide sequence identified as "NT SEQ ID NO:X" was assembled from partially homologous ("overlapping") sequences obtained from the 'cDNA clone ID" identified in Table 1 and ...[t]he cDNA Clone ID was deposited on the date and given the corresponding deposit number listed in "ATCC Deposit No:Z and Date."

See, specification page 69, lines 2-9.

Additionally, the specification describes the rationale for directing claims to the HEMCM42 cDNA of the ATCC Deposit and a particular SEQ ID NO:X or Y obtained from the deposit as follows:

> DNA sequences generated by sequencing reactions can contain sequencing errors...Accordingly, for those applications requiring precision in the nucleotide sequence or the amino acid sequence, the present invention provides not only the generated nucleotide sequence identified as SEQ ID NO:X and the predicted translated amino acid sequence identified as SEQ ID NO:Y, but also a sample of plasmid DNA containing a human cDNA of the invention deposited with the ATCC, as set forth in Table 1.

See Id. at page 70, lines 1-14. Indeed, the M.P.E.P., in recognition of the difficulties associated with the enablement of a biological material, states that "where the invention involves a biological material and words alone cannot sufficiently describe how to make and use the invention in a reproducible manner, access to the biological material may be necessary for the satisfaction of the statutory requirements for patentability under 35 U.S.C. § 112." (M.P.E.P. 2402). In view of the above, Applicants submit that the present specification provides sufficiently ample guidance to enable one of skill in the art to make and use the cDNA HEMCM42 commensurate in scope with the presently claimed antibodies.

Additionally, the Examiner alleges that "transformation of the material contained in ATCC Deposit Number 209075 into bacteria will allow for the selection of bacteria that contain plasmid(s) marked with the antibiotic resistance gene, but will not allow for the

direct selection of HEMCM42 per se." *See*, Paper No. 08232004, page 8, first paragraph. Further, the Examiner alleges that:

> [s]tandard library screening techniques disclosed in the specification (page 105, lines 23-36) may not be a reliable method to isolate HEMCM42, since a probe would need to be generated based on SEQ ID NO:13, a sequence that may be different from the claimed invention

and

> [t]he alternative disclosed method, using PCR to isolate HEMCM42 (page 106, lines 1-13), would also be problematic since PCR can introduce mutations as part of the amplification process.

Id. at page 8, second and third paragraphs.

Applicants respectfully disagree and assert that the methods disclosed in the specification were routine for a person having ordinary skill in the art as of the priority date of the present invention and consequently would not require undue experimentation for the isolation of a particular clone from the deposited sample. Furthermore, according to the M.P.E.P. § 2164.01(b), "[a]s long as the specification discloses at least one method for making and using the claimed invention that bears a reasonable correlation to the entire scope of the claim, then the enablement requirement is satisfied." Citing *In re Fisher*, 427, F.2d 833, 839 166 U.S.P.Q. 18, 24 (C.C.P.A. 1970).

In the instant case, the Examiner appears to be arguing that SEQ ID NO:23 would not be an ineffective tool for isolating the HEMCM42 cDNA because it may be different from the claimed invention. However, as discussed above, the specification discloses that the nucleotide sequence disclosed as SEQ ID NO:23 was determined from the HEMCM42 cDNA and both SEQ ID NO:23 and the HEMCM42 cDNA represent the same polynucleotides encoded by Gene No. 13. Therefore, the screening method disclosed in the specification using a radiolabelled probe based off of SEQ ID NO:23 and/or the PCR method using 17-20 nucleotide primers from the 5′ and 3′ ends of SEQ ID NO:23 *would* allow one of skill in the art to successfully isolate the HEMCM42 cDNA clone from the other plasmids in the ATCC Deposit. *See*, specification, Example 1, page 105, line 23 to page 106, line 13.

Furthermore, following either method, sequencing of the isolated clone by known sequencing methods or by restriction enzyme analysis would confirm that the DNA sequence obtained is representative of the HEMCM42 cDNA and SEQ ID NO:23

nucleotides of Gene No. 13. Moreover, confirmation of the sequence would preclude the possibility of isolating a clone that "may contain additional plasmids that encode different polypeptides yet have the same antibiotic resistance marker" and the possibility that "this plasmid could be lost even though the bacterial maintain their antibiotic resistance." Paper No. 08232004, page 8, first and second paragraphs.

Thus, Applicants submit that due to: (1) the amendments of claims 38 and 61 to recite "wherein said polypeptide encoded by the HEMCM42 cDNA is at least 95% identical to SEQ ID NO:59;" (2) the disclosure of the relationship between the HEMCM42 cDNA and SEQ ID NO:23 in the specification; (3) the availability of the deposit (as discussed below); (4) the well-established methods taught in the specification or from otherwise routine methods known in the art for isolating cDNA from a plasmid; and (5) the high level of skill in the art of molecular biology, it would not require undue experimentation to make and use the HEMCM42 cDNA deposit commensurate in scope with the pending claims. Accordingly, Applicants respectfully request the Examiner to reconsider and withdraw the rejection of claims 38-75 under 35 U.S.C. § 112, first paragraph for alleged lack of enablement.

Availability of the Deposit

The Examiner requested that applicants assure public availability of the biological material deposited in connection with the present application. To comply with the Examiner's request Applicants herein provide the following affirmation:

> Human Genome Sciences, Inc., the assignee of the present application, has deposited biological material under the terms of the Budapest Treaty on the International Recognition of the Deposit of Micro-organisms for the Purposes of Patent Procedure with the following International Depository Authority: American Type Culture Collection (ATCC™), 10801 University Boulevard, Manassas, Virginia 20110-2209 (present address). The deposit was made on May 22, 1997, accepted by the ATCC™, and given ATCC™ Accession Number 209075. In accordance with M.P.E.P. § 2410.01 and 37 C.F.R. § 1.808, assurance is hereby given that all restrictions on the availability to the public of ATCC™ Accession Number 209075 will be irrevocably removed upon the grant of a patent based on the instant application, except as permitted under 37 C.F.R. § 1.808(b). The assignee of the present application has been notified of its responsibility to replace the deposited biological material should the deposited material be destroyed or rendered non-viable.

In view of the above affirmation and explanation, attested to by the signature (below) of the Agent for the Applicants, it is respectfully requested that the rejection of claims 38-53 and 61-75 under 35 U.S.C. § 112, first paragraph, be withdrawn.

C. Claims 15-23, 36, 37, 52-60, 74, and 75

Claims 15-23, 36, 37, 52-60, 74, and 75 have been rejected under 35 U.S.C. § 112, first paragraph for alleged lack of enablement. In particular, it was alleged that while the specification is "enabling for an antibody obtained from an immunized animal, or an isolated cell or a hybridoma that produces an antibody" the specification is allegedly not enabling "for an immunized animal, an isolated cell or a hybridoma that makes a fragment of an antibody." *See,* Paper No. 08232004, page 10, paragraph no. 9.

Preliminarily, Applicants point out that without aqcuiescing to the present rejection, claims 16-23, 37, 53-60, and 75 (relating to hybridoma cells and immunized animals) have been canceled without prejudice or disclaimer. Therefore, the rejection as it relates to claims 16-23, and 53-60 under 35 U.S.C. § 112, first paragraph has been rendered moot. Applicants reserve the right to pursue subject matter encompassed by all canceled claims in one or more divisional or continuation applications.

With respect to the remaining rejected claims (drawn to isolated cells that produce an antibody or fragment thereof), Applicants respectfully disagree and traverse. Applicants submit that provided the disclosure of the present application and given the state of the art as of the earliest claimed benefit date, a person of ordinary skill in the art was enabled to produce the claimed antibody fragments directly from many types of isolated cells. To exemplify a few cell types that could have been used, Applicants submit herewith the following publications:

Exhibit A: Winter, et al., "Making Antibodies by Phage Display Technology", Ann. Rev. Immunol., vol. 12, pp. 433-455 (1994) (describing antibody fragments expressed on the surface of bacteriophage reproduced in infected bacteria);

Exhibit B: Forsberg, et al., "Identification of Framework Residues in a Secreted Recombinant Antibody Fragment That Control Production Level and Localization in *Escherichia coli*", J. Biol. Chem., vol. 272, No. 19, pp.12430-12436 (May 9, 1997) (describing production of an Fab fusion protein in *E. coli*); and,

Exhibit C: Abrams, et al., "Determinants of Specificity of a Baculovirus-expressed Antibody Fab Fragment That Binds Selectively to the Activated Form of Integrin $\alpha_{IIb}\beta_3$",

J. Biol. Chem., vol. 269, no. 29, (July 22, 1994) (describing production of an antibody fragment in insect cells).

In view of the above examples and evidence, Applicants assert that the pending claims meet the enablement requirements of 35 U.S.C. § 112, first paragraph. Thus, Applicants respectfully request that the instant rejection of pending claims 15, 36, 52, and 74 be reconsidered and withdrawn.

D. Claims 17-23 and 54-60

Claims 17-23 and 54-60 were rejected under 35 U.S.C. § 112, first paragraph. *See,* Paper No. 08232004, page 11, paragraph no. 10. Without acquiescing to the present rejection, Applicants have herein canceled claims 17-23, and 54-60 without prejudice or disclaimer. According, the rejection of these claims has been rendered moot.

Allowed Claims

The Examiner has indicated that claims 1-10, 13, 14, 24-31, 34, and 35 are allowable. *See,* Paper No. 08232004, page 15, paragraph no. 11.

Conclusion

Applicants thank the Examiner for his thoughtful consideration of the present application. Applicants respectfully request that the above-made remarks be entered and made of record in the file history of the instant application. The Examiner is invited to call the undersigned at the phone number provided below if any further action by Applicant would expedite the examination of this application.

If there are any fees, not already accounted for, due in connection with the filing of this paper, please charge such fees to our Deposit Account No. 08-3425.

Dated: Dec. 8, 2004

Respectfully submitted,

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MAKING ANTIBODIES BY PHAGE DISPLAY TECHNOLOGY

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Abstract

Antibody fragments of predetermined binding specificity have recently been constructed from repertoires of antibody V genes, bypassing hybridoma technology and even immunization. The V gene repertoires are harvested from populations of lymphocytes, or assembled in vitro, and cloned for display of associated heavy and light chain variable domains on the surface of filamentous bacteriophage. Rare phage are selected from the repertoire by binding to antigen; soluble antibody fragments are expressed from infected bacteria; and the affinity of binding of selected antibodies is improved by mutation. The process mimics immune selection, and antibodies with many different binding specificities have been isolated from the same phage repertoire. Thus human antibody fragments have been isolated with specificities against both foreign and self antigens, including haptens, carbohydrates, secreted and cell surface proteins, viral coat proteins, and intracellular antigens from the lumen of the endoplasmic reticulum and the nucleus. Such antibodies have potential as reagents for research and in therapy.

INTRODUCTION

In the immune system, the rearrangement of the V gene segments creates a repertoire of virgin B cells, each displaying a single antibody species.

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Cells are selected by encounter and binding of antigen, and they are triggered to differentiate to short-lived plasma cells that secrete antibody and to long-lived memory cells that persist in lymph nodes, spleen, and bone marrow. The V genes of the selected antibodies displayed on memory cells are subject to hypermutation, leading to antibodies of improved binding affinity after further selection with antigen. Thus repeated immunization leads to "affinity maturation" of the response (Figure 1). The immortalization of antigen-stimulated B cells by fusion to myeloma cells (1) taps the immune repertoire and has led to a wealth of rodent monoclonal antibodies with predefined specificity.

Technologies have been emerging for making antibodies in vitro by mimicking the selection strategies of the immune system (2–4). Repertoires of antibody fragments are displayed on the surface of filamentous bacteriophage, each displaying a single antibody species; the phage are selected by binding to antigen; and finally soluble antibody fragments are secreted from infected bacteria (Figure 1). As in the immune system, the V genes can be subjected to random mutation, and mutants may be selected with higher binding affinities. This allows the isolation of human antibody fragments of defined specificity, against both foreign and self-antigens. The technology is evolving fast (reviewed in 5–7), and here we review recent progress.

TECHNOLOGIES FOR SELECTION

Mimicking the B Cell

In the immune system, the B cell represents a self-replicating package containing the antibody genes that encode the antibody displayed at its surface. Phage display mimicks the B cell. Filamentous phage was first used to display small peptides by fusion to the minor coat protein (pIII: probably three or five copies per phage particle; here illustrated with three copies) (8). Two sites of pIII were used for fusion: in the flexible spacer between the two domains of pIII (8), or close to the N-terminus (9) or at the N-terminus (10). The phage were enriched by binding of peptide to monoclonal antibody. Through growth of the enriched phage and further selection by binding to antibody, very rare phage could be isolated (8).

Surprisingly, folded antibody fragments (2) and other proteins (11, 12) can also be displayed on phage. The antibody fragments can be displayed as single chain Fv fragments, in which VH and VL domains are connected on the same polypeptide chain by a flexible polypeptide spacer (13, 14), or as Fab fragments, in which one chain is fused to pIII and the other is secreted into the periplasm (2, 15–18). When antibody fragments are fused to the N-terminus of pIII, the phage is infective (2, 15). However, if the

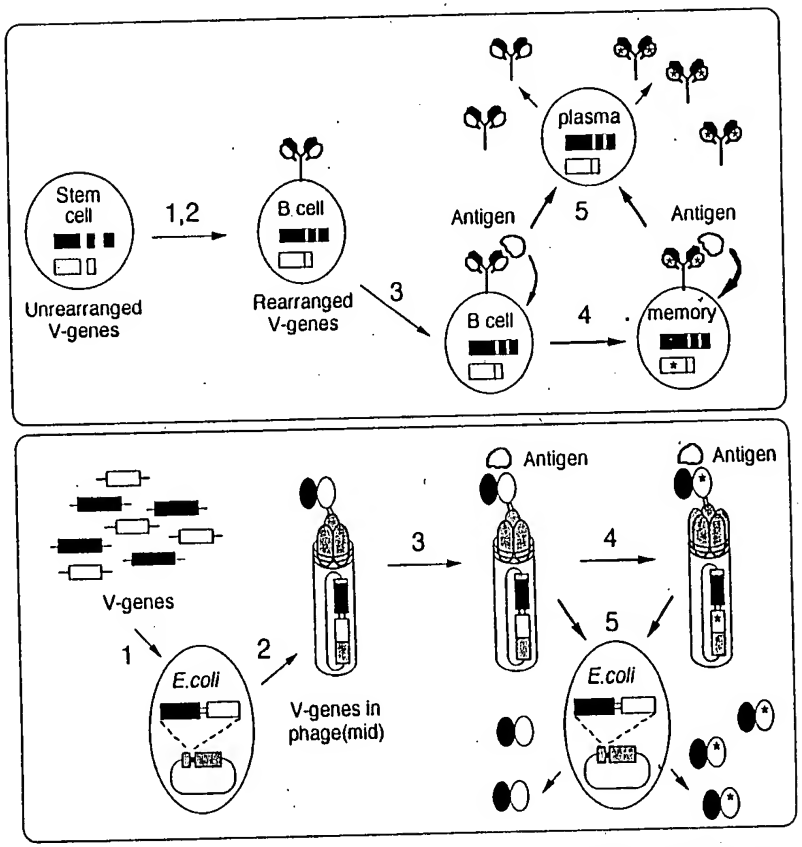

Figure 1 Generation of antibodies by the immune system and phage technology. Steps: (1) rearrangement or assembly of germline V genes; (2) surface display of antibody (fragment); (3) antigen-driven or affinity selection; (4) affinity maturation; (5) production of soluble antibody (fragment).

N-terminal domain of pIII is excised and fusions made to the second domain, the phage is not infective, and wild type pIII must be provided by helper phage (see below) (11, 16, 17) (Figure 2).

The pIII fusion and other proteins of the phage can be encoded entirely within the same phage replicon (2, 8), or on different replicons (11, 15–19). When two replicons are used, the pIII fusion is encoded on a phagemid, a plasmid containing a phage origin of replication. Phagemids can be packaged into phage particles by "rescue" with a helper phage such as

Figure 2 Display of antibody domains as pIII and pVIII fusions using phage and phagemid vectors. Antibody domains are depicted as black (heavy chain, VH or VHCH1) or white (light chain, VL or VLCL) spheroids; the genes are marked in similar fashion. Depicted are pIII fusion as phage (2) or phagemid (15) (18); truncated pIII fusion as phagemid (16, 17); pVIII fusion as phage (28, 30) or phagemid (27, 29). Only infectious phage particles displaying antibody domains are shown.

M13K07 that provides all the phage proteins, including pIII, but due to a defective origin is itself poorly packaged in competition with the phagemids (20).

The pIII fusion is often proteolysed, as shown by gel electrophoresis of the phage proteins and detection with anti-pIII antisera (J McCafferty, unpublished data). This is expected to give a population of phage particles, each displaying zero, one, two, three (and perhaps four and five) antibody fragments. The average valency of the population is further reduced by use of helper phage, in which the helper pIII competes for incorporation into the phage particle. Such phage have been estimated on average to display less than a single fusion protein per particle; they have been termed "monovalent" phage (17, 21). Other helper phages (M13ΔgIII) that lack pIII have been designed to rescue phage particles that incorporate only the pIII fusion from the phagemid; these are therefore multivalent (22). Use of different helpers can thereby alter the valency of the phages.

The major coat protein of the phage (pVIII: 3000 copies per phage particle) can also be used to display peptides (23–26) and antibody fragments (27–30). Pentapeptides (23, 24) and hexapeptides (25) were fused close to the N-terminus of pVIII, but phage encoding longer peptides were not viable unless wild type pVIII was provided (25, 26). The phage population is multivalent. With helper pVIII, up to about 900 peptides (25) and 24 antibody fragments (27) are incorporated per phage particle.

Fusions to pIII rather than pVIII have to date been preferred for antibody display.

Mimicking Immune Selection

In the immune system, encounter with antigen involves triggering the B cell through its receptor, and proliferation and differentiation to produce plasma cells that secrete antibody (reviewed in 31). The process appears capable of selecting one or more B cells from repertoires of $< 5 \times 10^8$ cells in mice and $< 10^{12}$ cells in humans (for review, see 32). Furthermore the immune system is able to selectively enrich for B cells displaying antibodies with slightly improved binding affinities, allowing affinities to be built up in a step-wise manner through rounds of mutation and selection (33).

Phage selection appears to be at least as powerful as immune selection. Phage displaying small peptides can be selected by direct binding to solid phase antibody (8), and also by binding to a biotinylated antibody in solution, which is then captured onto solid phase streptavidin (9). Likewise phages displaying antibodies can be selected by binding to antigen coated plates (16, 34), column matrices (2), cells (35), or to biotinylated antigen in solution followed by capture (36). The phages bound to the solid phase are washed and then eluted by soluble hapten (37), acid (16) or alkali (34). Phages can be enriched 20–1000 fold by a single round of selection (2, 17, 34). Moreover, the enriched phages can be grown in bacterial culture and subjected to further rounds of selection. In this way, enrichment factors of only 50-fold in each round can build up to 10^7-fold enrichments over four rounds of selection (34).

SELECTION EFFICIENCY The efficiency of selection is likely to depend on many factors, including the kinetics of dissociation during washing, and whether multiple antibody fragments on a single phage can simultaneously engage with (solid phase) antigen. For example, antibodies with fast dissociation kinetics (and weak binding affinities) should be retained by use of short washes, multivalent display and a high coating density of antigen at the solid phase. The high density should not only stabilize the phage through multivalent interactions, but favor rebinding of phage that has dissociated. Nevertheless, it appears that binding affinities (for a single antibody fragment) of 10^5 M^{-1} are barely sufficient to hold multivalent phage to solid phase (37).

Conversely the selection of antibodies with slow dissociation kinetics (and good binding affinities) should be promoted by use of long washes (11), monovalent phages (11), and a low coating density of antigen (38). In principle, phages with very high affinities ($> 10^{10}$ M^{-1}) should be difficult to elute, but a change in pH may suffice to dissociate the complex (21, 39);

the phage also survive 5M guanidine hydrochloride (M Figini, unpublished data).

DISCRIMINATION In immune selection, the virgin B cells displaying antibodies with (unwanted) self-specificities are deleted or rendered anergic (40). With phage, it has proved more difficult to deplete the repertoire, for example by preabsorption, as it is difficult to capture all the phage that can bind, and many of the phages are "bald," lacking antibody fragments due to proteolysis. Nevertheless, preabsorption (on red blood cells lacking the blood group E antigen) was used for isolation of phage specificities against the blood group E antigen (35).

As with immune selection, it is also possible to select between phage antibodies of different affinities (37), even with affinities that differ slightly (36). In the later immune response, B cells are thought to compete for limiting antigen in the germinal centres (for review, see 41). Likewise in selection of peptide phages with biotinylated antibody (9), limiting antibody was used to promote competition between the phages (42).

However, random mutation of a selected antibody is likely to give rise to many mutants, most binding to antigen, and a few with higher affinity. With limiting antigen, rare high affinity phage could be competed out. To retain all the higher affinity mutants, phages can be incubated with excess soluble biotinylated antigen, but with the antigen at a lower concentration than the target affinity constant. The phages are then captured by streptavidin-coated paramagnetic beads. Such "equilibrium capture" allows the antibodies to be selected according to their affinities of binding, provided no two antibody fragments on the same phage bind to the same molecule of antigen. Using this technique, mutant phage antibodies have been selected from a great excess of phages with two- to four-fold lower affinities over many rounds of selection (36, 112).

Discrimination can be enhanced by taking advantage of dissociation kinetics. Thus for two phages dissociating from antigen with slightly different kinetics, the discrimination should increase with time due to the exponential nature of the decay. Indeed this was demonstrated by dissociation of phages from biotinylated antigen in solution (36). Using such kinetic selection, even mutant antibodies with a two-fold higher affinity could be selected from a great excess of phages with lower affinity (112). Washing of phages bound to a solid phase should also discriminate by dissociation kinetics.

Discrimination may be compromised by multivalent interactions (10, 11, 16, 21), but this will depend on the affinities, kinetics, and the selection process. Multiple interactions increase the avidity of phage binding, and slight differences in affinity between two antibodies should in fact give

rise to greater differences in avidity between the two phages, potentially enhancing discrimination. However, if the avidities became so strong that both phages bound very tightly to the solid phase antigen, discrimination would be lost, especially with low stringency washes.

Mimicking the Plasma Cell

Antibody fragments can be characterized and used as free soluble fragments or as phage. Binding can be detected by ELISA using antisera against the phage (2); the affinity of binding can be measured with soluble radioactive antigen (17); and dissociation kinetics by loss of phage from its complex with biotinylated antigen (36, 112). Furthermore, phages displaying antibody fragments can be used as reagents in Western blots, and for fluorescence staining of cells (A Nissim, unpublished data).

Phagemid vectors can also be engineered for display or for secretion of free antibody fragments from infected bacteria. By incorporating an amber stop codon between the fragment and pIII, the antibody fragments are fused to pIII and displayed when the amber codon is suppressed, and secreted when it is not (15). The growth of phage in suppressor and nonsuppressor bacteria therefore mimics respectively the surface display of antibodies on B cells, and the production of fragments from plasma cells (Figure 3). The same approach was used for display and secretion of human growth hormone (21). Less conveniently, the V genes encoding antibody fragments can be recloned for secretion (16, 37).

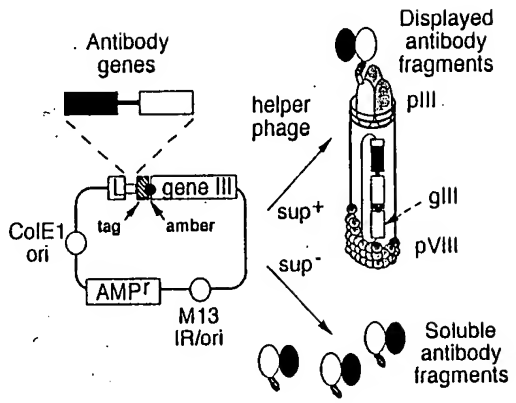

Figure 3 Mimicking the plasma cell. Phagemid pHEN1 (15) allows antibody domains to be displayed on phage after rescue with helper phage from an E. coli suppressor strain, or the domains to be secreted as (tagged) soluble fragments from non-suppressor strains. AMP = ampicillin resistance gene, L = leader peptide sequence, tag = c-myc peptide sequence.

Antibody fragments can be secreted from bacteria with yields ranging from 0.2–2 mg/l fragments in shaker flasks (43–45), or >500 mg/l in fermenters (46); and they can be harvested from the culture supernatant (44) or the periplasm (43). Protein A has been used to purify antibody fragments of the human VHIII family (47), and protein G to purify Fab fragments by binding to the CH1 domain (48). Engineered C- or N-terminal peptide tags that bind to monoclonal antibodies (49, 50) or to streptavidin (51) have also been used for both purification and detection of antibody fragments, but hexahistidine tags binding to immobilized metal chelate groups (52) seem particularly valuable for purification (53).

Antibody fragments can be characterized on a solid phase or in solution. Attempts have been made to measure binding affinities by competition ELISA (54), but the method is only qualitative (55) and may be more suitable for ranking of binding affinities. Even so this assumes no aggregation or dimerization. Thus, the reported in vitro affinity maturation of antibody fragments (56) could have been due to dimerization of the scFv fragments (57, 58). A more rigorous ELISA method (59) based on equilibrium capture would have been more suitable (55). Antibody fragments have also been characterized by binding to an antigen-coated surface by surface plasmon resonance (38, 57, 60). However, account needs to be taken of the fraction of active antibody (for determination of association rates), and of dimerization and of rebinding to the highly coated surface (for determination of dissociation rates) (61). For measurement of affinities in solution, the use of fluorescence quench titrations is often suitable for haptens (37, 62), but it is more difficult for protein antigens unless there is a large quench on binding (63).

TECHNOLOGIES FOR MAKING V-GENE REPERTOIRES

Diversity of Antibody Sequences and Structure

In the immune system the sequence diversity of antibody binding sites is not encoded directly in the germline but is assembled in a combinatorial manner from V gene segments. In human heavy chains, the first two hypervariable loops (H1 and H2) are drawn from <50 VH gene segments (64), which are combined with D segments and JH segments (65) to create the third hypervariable loop (H3). This loop is exceptionally variable in sequence and length (2–26 residues) (66); because the joining of the segments is imprecise, different reading frames of the D segment may be used, nucleotides can be inserted and deleted at the junctions, and the D segments can recombine as D-D fusions (67).

In human light chains, the first two hypervariable loops (L1 and L2) and much of the third (L3) are drawn from probably <30 Vλ (68) and <30 Vκ gene segments (JPL Cox, IM Tomlinson, unpublished data). These segments are combined with Jλ and Jκ segments to complete the third hypervariable loop (L3). This loop has limited variability. It ranges in size from 7 to 11 residues in λ light chains (69) and is most commonly 6 residues in κ light chains (70) but can vary between 5 and 8 residues (71). Thus, most of the sequence diversity (and structural diversity—see below) is encoded by the heavy chains.

Despite the immense sequence diversity, most of the loop conformations of antibody binding sites are relatively conserved (72–74). Implicit in the sequences of the VH germline segments are three major conformations for the H1 loop and five for the H2 loop. In combination they provide seven different folds (74). By contrast, the H3 loop of the rearranged heavy chains is likely to provide a huge range of structures. Implicit in the sequences of the Vλ segments are at least three major conformations for the L1 loop and at least two for the L2 loop (68). In the Vκ segments, there are probably four major conformations for the L1 loop and one for the L2 loop; in combination these provide four different folds (JPL Cox, IM Tomlinson, unpublished data). The combinations of different loops, decorated with side chains, create a wealth of binding sites ranging from flat surfaces (75) to pockets (76).

The potential diversity of different sequences in the primary immune repertoire is far greater than the number of B cells at any time. However, some sequences may not fold, and others may produce identical loop conformations: the repertoire of binding site structures is likely therefore to be much smaller than the sequence repertoire. Presumably the V gene segments and their representation in the expressed antibody repertoire reflect the efforts of the immune system over evolution to encode a diverse structural repertoire with a limited number of B cells. For phage repertoires, the V gene segments appear therefore to be suitable building blocks for making a diverse repertoire of structures.

Repertoires of VH and VL genes

The use of the polymerase chain reaction, with primers matching the 5′ and 3′ ends of rearranged VH and VL genes, provided the means to amplify, clone, and express V genes from lymphocytes (77), thereby making diverse V gene repertoires for expression (Figure 4). The V genes may be amplified from both cDNA and genomic DNA, with back primers at the 5′ end of the exon encoding the mature V-domain and forward primers based within the J-segment (49, 77). However, for amplifying from cDNA, "back" primers have also been based in the leader exon (79), and forward primers within the constant region (78). To maximize complementarity,

degeneracy was incorporated into the primers (77, 78), or different primers were designed for different families of V genes (80). For cloning of the amplified DNA into expression vectors, rare restriction sites were introduced within the PCR primer (77), as a "tag" at one end, or by further PCR amplification with a tagged primer (37). "Primary" repertoires of V genes harvested from a lymphocyte population are likely to contain somatic mutations, although most published human VH and Vκ gene sequences encode few (<5) amino acid substitutions (64; JPL Cox, IM Tomlinson, unpublished data).

Repertoires of "synthetic" rearranged V genes have also been derived in vitro from V gene segments (Figure 4). Most of the human VH-gene segments have now been cloned, sequenced (64), and mapped (81); these cloned segments (including all the major conformations of the H1 and H2 loop) have been used to generate diverse VH gene repertoires with PCR primers encoding H3 loops of diverse sequence and length (47; A Nissim, unpublished data). VH repertoires have also been made with all the sequence diversity focussed in a long H3 loop of a single length (82). Human Vκ and Vλ segments have been cloned and sequenced (68; JPL Cox, IM Tomlinson, unpublished) and are therefore available for making synthetic light chain repertoires. Synthetic V gene repertoires, based on a range of VH and VL folds, and L3 and H3 lengths, should encode antibodies of considerable structural diversity.

Combining VH and VL Gene Repertoires

Most of the structural diversity of antibody binding sites appears to be contributed by heavy rather than light chains (see above). Indeed, heavy

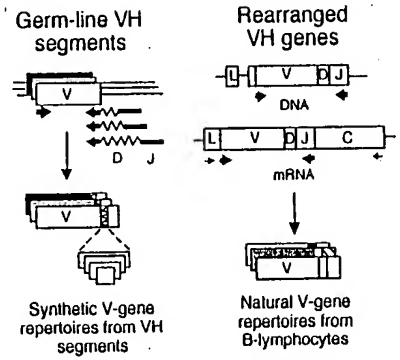

Figure 4 Generation of V gene repertoires. The location of the primers for PCR amplification of V gene repertoires from segments (47) or from rearranged V genes (34, 37) are indicated.

chains and VH domains (49) have been found with binding activities in the absence of the light chain. Furthermore in camels, two of the heavy chain isotypes lack the CH1 domain and do not appear to associate with light chains (83). However, the structures of complexes of antibody and antigen indicate that usually both domains make important interactions (75, 84–86). Presumably the role of VL domains is to add structural diversity, for example, in helping to make binding clefts, and to create a larger surface of interaction with antigen. Both features should enhance the probability of finding an antibody that binds to antigen with good affinity.

Repertoires of antibody fragments have been constructed by combining VH and VL gene repertoires together in several ways (Figure 5). Each repertoire can be created in different vectors, and the vectors recombined in vitro (87) or in vivo (88); alternatively, the repertoires may be cloned sequentially into the same vector (16) or assembled together by PCR and then cloned (37). A technique of "in-cell PCR assembly" has also been described for combining the VH and VL genes within the lymphocyte by PCR, and then cloning the repertoires of linked genes (89). Repertoires of VH domains have also been combined with a single VL gene (47, 82). The route by which repertoires are combined can dictate the structural diversity and repertoire size. For example, combining VH and VL repertoires in vivo, by combinatorial infection (88) (see below), should allow the creation of libraries of $>10^{12}$ different VH/VL combinations.

ANTIBODIES MADE FROM PHAGE DISPLAY

Taking Advantage of Immunization

Immunization leads to an increase in the number of cells making an immune response, but especially in the levels of mRNA. Resting B cells make about 100 copies of Ig mRNA per cell, whereas a hybridoma (and also presumably a plasma cell) makes about 30,000 copies (90). Spleen, lymph nodes, tonsils, and bone marrow (but not peripheral blood lymphocytes) provide a rich source of plasma cells and Ig mRNA. Repertoires of VH or VL genes amplified from the mRNA of spleen cells of an immunized mouse are therefore greatly enriched in V genes encoding part of an antigen binding site (91).

In random combinatorial libraries (48), the VH and VL gene repertoires are combined at random, and the original combinations of the immune lymphocyte are destroyed. Nevertheless, if the V gene repertoires are derived from the mRNA of lymphocytes after immunization, antigen binding fragments are created at low frequency, at best <1/500 (92), and more usually <1/5000 (93, 94). The power of phage selection allows

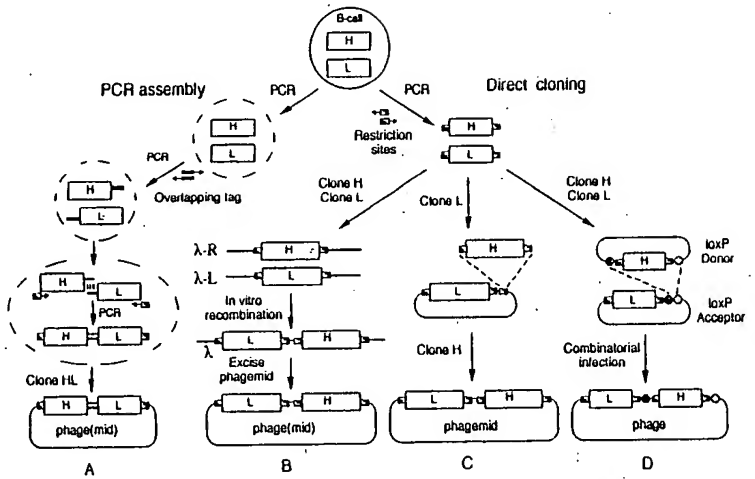

Figure 5 Linking the V genes together. (A) PCR assembly allows a one-step cloning of heavy (H) and light (L) DNA in scrambled pairings (34, 37), or original pairings if "in-cell" (89). Alternatively (B, C, D) heavy (H) and light (L) chain DNA is cloned separately and combined by in vitro recombination (B) (87), or combinatorial infection (D) (88), or cloned sequentially (C) (16).

many of these fragments to be isolated and characterized. For example, a repertoire of antibody fragments was assembled for phage display from the mRNA of mouse splenocytes after immunization with the hapten phenyloxazolone (phOx). The VH and Vκ genes encoding a range of fragments were found to be similar to those of hybridomas of the phOx response, but in general the pairings were not (37, 95).

Furthermore, as suggested by λ phage combinatorial libraries (93, 94), the pairings were promiscuous, that is, the same light chain could be found with different heavy chains, and vice versa. By "shuffling" a promiscuous heavy chain with the repertoire of light chains, a further range of partners were found for binding to phOx (37). Combinatorial repertoires from immunized sources therefore appear to be dominated by "artificial" pairings, as predicted (4). Although original pairings are likely to be present in large random combinatorial libraries, it is impossible to distinguish original from artificial pairings. However, it may be possible to determine these pairings by first linking the VH and VL genes within the lymphocyte (89)

Nevertheless, the artificial pairings from phage display libraries and, enriched by immunization, can provide antibody fragments with good affinities. For example, an antibody fragment isolated from the phOx

response (as above) had a binding affinity of 10^8 M^{-1} for hapten, with every prospect that higher affinity antibodies were present in the repertoire (37). This compares with typical affinities of 10^8 M^{-1} for secondary phOx antibodies from hybridomas (62, 96), and with affinities of 7.5×10^6–4×10^8 M^{-1} for hybridomas isolated from the same immunized spleen (95).

Antibody fragments have also been isolated from immunized humans with binding activities against several viral antigens, for example, HIV gp120 (54, 97, 98), respiratory syncytial virus (RSV) (99), and hepatitis B virus (100). The fragments against HIV and RSV were capable of neutralizing virus infection (97, 99). Furthermore, specificities against herpes simplex virus, human cytomegalovirus, varicella zoster virus, rubella, RSV, and HIV were derived from the same V gene repertoire from a patient immune to these pathogens (101). Extensive chain promiscuity has also been seen for human antibody fragments derived from combinatorial libraries directed against HIV gp 120: it was argued that the heavy chains must have arisen from antigen-specific clones in vivo (98).

By-Passing Immunization

As the display and selection of antibodies on phage mimicks immune selection, it should be possible to isolate antibody fragments of any required specificity directly from a single phage repertoire of sufficient size and diversity. Importantly, it should provide antibody specificities directed against self-antigens that are difficult to raise by immunization, owing to tolerance mechanisms.

NATURAL REPERTOIRES A diverse source of rearranged V genes was provided by human peripheral blood lymphocytes (PBLs), using "family-based" PCR primers to amplify each of the human VH, Vκ, and Vλ families (80). The repertoires of VH and VL genes were combined at random, as this should destroy the original combinations and specificities of the PBLs and generate new specificities (34).

From this library, it was possible to isolate phage with binding activities against many different antigens. For example, antibodies were isolated against the foreign antigens bovine serum albumin (BSA), turkey egg lysozyme, the hapten phOx (34), and bovine thyroglobulin (57), and against the human self-antigens tumor necrosis factor α (TNFα), thyroglobulin, a monoclonal antibody, carcinoembryonic antigen (CEA), mucin and CD4 (57). Antibody fragments against the monoclonal antibody mapped to both variable and constant regions (57). Antibodies were also isolated against the human blood group antigens of the ABO and I blood group systems (B and HI), of the Rh system (D and E), and of the Kell system (Kpb) (35). For the anti-blood groups, the selections were

undertaken by binding the phage to red blood cells; the anti-E phage was only selected after first preabsorbing the phage library with red cells lacking this antigen.

The antibodies from the library were shown to be highly specific by screening for binding to a panel of other antigens (34, 57). Specificity was also demonstrated by the staining of kidney sections with the anti-B: the only cells stained were the endothelial cells bearing the blood group B antigen (35). The affinities of the antibodies were typical of a primary immune response, in the range 10^5 M^{-1}–10^7 M^{-1}, but dimerization of the scFv fragments led to improved avidities (57). Antibody fragments were also derived from V genes prepared from unimmunized rodent bone marrow. However, the library was selected only against the hapten progesterone, the binding affinities were poor (apparently 10^4–10^5 M^{-1} by competition ELISA), and the fragments cross-reacted with another protein (56).

Although a range of anti-self specificities can be derived from a "single pot" library from "natural" rearranged V genes, it is impossible to prove that one or another of the antibody chains was not derived from B cells with self-specificity. Moreover, in most cases the sequences of both chains were somatically mutated, suggesting that the chains were derived from an antigen-driven process (35, 57); indeed and for the anti-blood group B specificities, anti-B could be detected in the donor antiserum (35).

SYNTHETIC REPERTOIRES Synthetic V gene repertoires can also be built from cloned human VH-gene segments. A repertoire (2×10^7 clones) was first constructed using a short H3 loop of five or eight random residues with each of 49 segments, and combined with a fixed light chain. Antibodies of high specificity were selected against two haptens, phOx and NIP (with affinities of up to 10^6 M^{-1}) and human TNF-α, but not against three other (protein) antigens (47). However, by adding a range of H3 loops of different lengths, up to 12 residues, a single library was created from which a range of more than 20 binding specificities could be selected, including against haptens; the foreign antigens lysozyme, keyhole limpet haemocyanin, streptavidin, and immunoglobulin binding protein (BIP); and the self-antigens the oncogene protein rhombotin and the tumor suppressor protein p53. The epitope of an antibody binding to p53 was mapped and found to be new. The antibodies appeared to be specific and could be used as reagents for immunofluorescence staining of p53 in the nuclei of cells, and for Western blotting of cell lysates for BIP (A Nissim, unpublished). This also illustrates that antibodies can be made against intracellular antigens, and in particular those of the lumen of the endoplasmic reticulum.

Other synthetic libraries have been built from the framework of a single

antibody. By randomizing the H3 loop a single binding specificity was selected against FITC (affinity 10^7 M^{-1}) (82); by randomizing the sequences of the L1, L3, H2, and H3 loops, a single binding specificity was selected against insulin-like growth factor (but not against CD4 or tissue plasminogen activator) (102). There clearly has to be sufficient structural diversity to make a working "single-pot" library.

MAKING HIGH AFFINITY ANTIBODIES

Mutation

For most purposes, antibodies must bind their antigen tightly. In the immune system, strong binding can be built from multiple weak interactions, as illustrated by the interactions of IgM with multivalent antigens such as virus. However, the higher affinity antibodies are made after repeated rounds of immunization, arising either as mutants of a primary response antibody, or as entirely new antibodies (repertoire: such antibodies may arise by somatic mutation of very low affinity antibodies (96). The increase in binding affinity of primary response antibodies is sometimes modest, with anti-NP hybridomas showing a five-fold improvement in affinity (103), or large, with anti-phOx hybridomas showing improvements of 100-fold (62). Site-directed mutagenesis of an anti-p-azophenylarsonate antibody also suggests that somatic mutation at a few sites can together contribute factors of >200 to binding affinity (104).

In phages, antibody fragments can be designed with higher binding avidities, for example, as single chain dimers (57) or "diabodies" (58). Presumably other multimeric fragments could be designed to mimic IgM. Furthermore, mutation can be introduced at random in vitro (36, 56) by using error prone polymerase (105), or in vivo by use of mutator strains of bacteria (106, 107), and the phage can be selected for higher affinities. However, the affinities of antibody fragments against a hapten and a protein antigen could be improved only a modest four-fold to 10^8 M^{-1} and 10^9 M^{-1}, respectively, using a single round of random mutation followed by multiple rounds of selection (36; RE Hawkins, SJ Russell, unpublished data). To make higher affinity mutants, it might be desirable to increase the frequency of random mutation or to combine rounds of mutation and selection, for example, by growing phage in bacterial mutator strains. Alternatively, it might be desirable to start with lower affinity antibodies (as may occur in repertoire shift), in the event that a higher affinity binding site is trapped at a local optimum and incapable of further affinity maturation (108).

Phage display appears to have potential advantages over the immune system for the creation of secondary (mutated) repertoires. Firstly, the size

of the secondary repertoires can be much larger than in immune systems. Secondly, random mutation can be focussed to the antigen binding loops or outside, for example, at framework residues that influence loop conformation (63). Indeed, mutations outside the contact surface with antigen can often have profound effects on binding affinity (104, 109, 112).

Chain Shuffling

In the immune system, somatic mutation of a selected pair of VH and VL domains appears to be the only mechanism for making structural variation of a selected antigen binding site. However, random combinatorial repertoires contain immense untapped diversity that can be mobilized by chain shuffling.

Chain shuffling was first used to analyze the promiscuity of VH and VL pairings in repertoires from immunized mice (37, 110). It was then used for the affinity maturation of a human antibody fragment (affinity 3×10^6 M^{-1}) for phOx isolated from a V gene repertoire. The VH gene was paired with VL genes from the original repertoire, and the new (light chain shuffled) repertoire was displayed on phage. A light chain partner was isolated that conferred improved binding affinity (6×10^7 M^{-1}). Likewise the new VL gene was paired with the original repertoire of VH genes, (but now combined with the H3 loop of the original VH gene), and after selection a fragment was isolated with a further improved affinity (10^9 M^{-1}). Indeed the affinities of the original and shuffled fragments are similar to those of mouse hybridomas of the primary and later responses to the same hapten.

In the high affinity fragment, both domains were derived from the same germline VH and VL genes as the parent, but with different patterns of mutations. The 20-residue changes suggest that large changes in affinity (500-fold here) might require many random mutations (38).

Chain shuffling can therefore be used to tap the somatically mutated V genes and make higher affinity binding sites. However, chain shuffling can also be used for more extensive diversification. For example, the heavy and light chains of mouse monoclonal antibodies against the hapten phOx (M Figini, unpublished) and human TNFα (H Hoogenboom, L Jespers, unpublished data) were sequentially replaced to create entirely human antibodies of the same specificity, a process termed *epitope imprinted selection*.

Large Repertoires

Theoretical studies have suggested, not surprisingly, that the larger the library, the greater the chance of finding antibodies that bind to any given epitope, and the higher the affinity (111). However, the limiting factor in

making large primary libraries is the efficiency of introduction of plasmid or phage DNA into bacteria. In practice, this limits the library size to 10^7–10^9 clones, even taking advantage of λ phage vectors with excisable filamentous phage replicons (87).

In principle, a simple way of increasing library size would be to generate more of the possible chain combinations. This has prompted a new approach—combinatorial infection (88). For example, if 10^5 different light chains were cloned for display as a Fab-pIII fusion in a phage vector, and then the phage used to infect $> 10^{12}$ bacteria harboring a library of 10^7 different heavy chains in a plasmid, this could create 10^{12} possible Fab fragments (15). If the two chains were recombined efficiently in vivo onto the same phage replicon by use of loxP sites (88), this would create a phage library with huge diversity. Indeed, it appears that such huge "teraphage" libraries can be created (88; AD Griffiths, P Waterhouse, unpublished), and this should allow high affinity antibodies to be isolated directly and indeed might also facilitate any chain shuffling required for further affinity improvements.

CONCLUSION

Phage display should facilitate the construction of human antibodies of therapeutic value and of research reagents. Libraries have been constructed that take advantage of immunization, or by-pass it, leading to antibodies with good binding affinities (10^8–10^9 M^{-1}) and high specificity against foreign and self-antigens. Targets have included viral coat proteins, BIP from the lumen of the endoplasmic reticulum, and surface markers of lymphocytes (T cell receptor and CD4), tumor cells (CEA and mucin) and red blood cells (B, D, E, I and Kell). The antibodies have been used to neutralize virus, to stain cells, and for Western blots.

There is clearly a future for "single pot" libraries, as the same library can be selected with a range of different antigens, and without the need for immunization of animals. The affinities of the antibodies isolated should improve as new technologies are used to increase the size and diversity of libraries. Indeed, the availability of the cloned human VH, Vκ, and Vλ gene segments, and knowledge about the structures they encode, should allow the design of maximum structural diversity in primary repertoires. It should also allow the creation of premutated genes for use in making secondary repertoires, in which mutations are focussed at the antigen contacts or at sites likely to modulate the contacts.

There may also be a future for "designer" libraries. As the potential antibody diversity is probably too large to be tapped in a single phage library, it may be advantageous to build libraries that are shaped for

complementarity to a defined antigen. As phage display can not only exploit the principles of immune selection, but also cannibalize and improve on the antibody building blocks, it should increasingly be capable of outperforming natural immune systems in making antibodies.

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Identification of Framework Residues in a Secreted Recombinant Antibody Fragment That Control Production Level and Localization in *Escherichia coli**

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The monoclonal antibody 5T4, directed against a human tumor-associated antigen, was expressed as a secreted Fab superantigen fusion protein in *Escherichia coli*. The product is a putative agent for immunotherapy of non-small cell lung cancer. During fermentation, most of the fusion protein leaked out from the periplasm to the growth medium at a level of approximately 40 mg/liter. This level was notably low compared with similar products containing identical C_H1, C_L, and superantigen moieties, and the Fv framework was therefore engineered. Using hybrid molecules, the light chain was found to limit high expression levels. Substituting five residues in V_L increased the level almost 15 times, exceeding 500 mg/liter in the growth medium. Here, the substitutions Phe-10 → Ser, Thr-45 → Lys, Thr-77 → Ser, and Leu-78 → Val were most powerful. In addition, replacing four V_H residues diminished cell lysis during fermentation. Thereby the product was preferentially located in the periplasm instead of the growth medium, and the total yield was more than 700 mg/liter. All engineered products retained a high affinity for the tumor-associated antigen. It is suggested that at least some of the identified framework residues generally have to be replaced to obtain high level production of recombinant Fab products in *E. coli*.

Antibody-based therapies are currently evaluated for treatment of several severe diseases such as cancer (1), viral infections, and autoimmunity. Recent technological improvements have made it possible to clone and produce large amounts of intact recombinant monoclonal antibodies or antibody fragments (2, 3). Using phage display technologies, high affinity antibodies can be obtained without prior immunization (4, 5). For production purposes *Escherichia coli* is a very useful host (reviewed in Ref. 6). Correctly folded Fab has been secreted at levels exceeding 1 g/liter (7) and single chain Fv molecules at slightly lower levels (8). Systems involving inclusion body formation and *in vitro* refolding have also been described (9, 10). Consequently, there are effective tools to obtain both these molecules and a variety of clinical applications.

Recently, a concept for cancer therapy using recombinant fusion proteins of tumor-reactive Fab fragment and immunostimulatory bacterial superantigens was presented (11, 12). Superantigens, such as the staphylococcal enterotoxin A (SEA),[1] activate cytotoxic and cytokine-producing T-lymphocytes. Antibody-targeted SEA can initiate a powerful T cell attack against tumor cells *in vivo* (12, 13).

Here *E. coli* production of the 5T4Fab-moiety fused to a genetically engineered superantigen chimera (14),[2] 5T4Fab-SEch, is investigated. The murine antibody 5T4 is directed against a trophoblast-related antigen found on several solid tumor types including carcinomas in lung, breast, colon, and ovary (16, 17). The 5T4Fab-SEch has a high affinity for the antigen and targets T cells to several cancer cell lines. However, when produced as a secreted fusion protein in *E. coli*, the production level is 5–10-fold lower compared with several similar products. To investigate the molecular components behind this phenomenon, several amino acid residues in the Fv framework were altered. Significantly, by replacing only a few light chain residues the level of active product increased, while heavy chain substitutions affected the product distribution between growth medium and periplasmic space.

EXPERIMENTAL PROCEDURES

Materials—Restriction endonucleases and *Taq* polymerase were from Boehringer Mannheim or New England Biolabs (Beverly, MA). The recombinant work was carried out mainly as described (18) in the *E. coli* strain HB101. Plasmid preparation was performed with Wizard® Midipreps DNA purification system (Promega, Madison, WI) from bacteria grown in LB medium with 50 μg/ml kanamycin. Oligonucleotides were synthesized on an ABI 392 DNA/RNA synthesizer (Applied Biosystems, Foster City, CA). Antibodies against murine κ chain were obtained from Bio-Zac (Stockholm, Sweden) and horseradish peroxidase-conjugated antibodies against SEA from Toxin Technology (Sarasota, FL).

Cloning, Engineering, and Insertion into an E. coli Expression Vector of 5T4 Fv—The Fv-encoding portions of 5T4 were cloned from the 5T4 hybridoma obtained from Dr. Peter Stern (CRCT, Paterson Inst. for Cancer Research, Manchester, UK). The cDNA was made from total RNA using the GeneAmp RNA PCR kit (Perkin-Elmer). The coding regions of the entire variable domains and parts of the signal sequences as well as the constant domains of the heavy and light chains were amplified by PCR. All PCR products and DNA linkers were sequenced on an ABI 373A DNA sequencer (Applied Biosystems) as recommended by the supplier. The oligonucleotides 5′-CAATTTTCTTGTCCACCTTGGTGC-3′ and 5′-ACTAGTCGACATGGGATGGAGCTITATCATI(C/T)TCTT-3′ were used for the heavy chain resulting in a 553-base pair fragment, while 5′-ACTAGTCGACATGGGCITCAAGATGGAGTCACA(G/T)(A/T)(C/T)(C/T)C(A/T)GG-3′ and 5′-GCGCCGTCTAGAATTAACACTCATTCCTGTTGAA-3′ were used for the light chain yielding a 724-base pair fragment. For each chain three separate clones were

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[1] The abbreviations used are: SEA, staphylococcal enterotoxin A; PCR, polymerase chain reaction; SEch, staphylococcal enterotoxin chimera; IPTG, isopropyl-β-D-thiogalactopyranoside; HPLC, high pressure liquid chromatography; CDR, complementarity-determining region.

[2] Antonsson, P., Gjörloff Wingren, A., Hansson, J., Kalland, T., Varga, M., and Dohlsten, M. (1997) *J. Immunol.*, in press.

sequenced and found to be identical. DNA fragments suitable for insertion into the expression vector (12) were obtained in a second PCR step. To assemble a Fab-expression plasmid, the variable regions of 5T4 were fused to sequences coding for the constant regions of the murine IgG1/k antibody C242 (12) and lacking the interchain disulfide bond. A region coding for a hybrid between SEA and staphylococcal enterotoxin E, SEA/E-BDEG,[2] with the substitution Asp-227 → Ala in the major histocompatibility complex II binding site (14), was connected to the C terminus of the heavy chain via a Gly-Gly-Pro linker. The 5T4 Fv sequence is shown in Fig. 1. All 5T4 mutants were made as one-chain constructs and combined with the partner chain in the expression plasmid. The point mutations coding for Phe-10 → Ser, Ile-63 → Ser, and Tyr-67 → Ser, as well as the heavy chain mutations were introduced by PCR while Thr-45 → Lys, Phe-73 → Leu, Thr-77 → Ser, Leu-78 → Val, and Leu-83 → Ala used synthesized oligonucleotide linkers. Gene segments containing the various point mutations were also combined (Table I). All constructs were verified by DNA sequencing.

Expression of 5T4Fab-SEch in the Fermenter—The products were expressed in the *E. coli* K-12 strain UL 635 (*xyl-7, ara-14,* $T4^R$, Δ*ompT*) using a plasmid with a kanamycin resistance gene and lacUV5 promoter. Bacteria from frozen stock were incubated at 25 °C for approximately 21 h in shaker flasks containing (per liter) 2.5 g of $(NH_4)_2SO_4$, 4.45 g of KH_2PO_4, 11.85 g of K_2HPO_4, 0.5 g of sodium citrate, 1 g of $MgSO_4 \cdot 7H_2O$, 11 g of glucose monohydrate, 0.11 mM kanamycin, and 1 ml of trace element solution (19), however, without $Na_2MoO_4 \cdot 2H_2O$. The cells were grown to an A_{600} of 1–2, and 450 ml of culture medium was used to inoculate a fermenter (Chemap, Switzerland) to a final volume of 5 liters. The fermenter medium contained (per liter) 2.5 g of $(NH_4)_2SO_4$, 9 g of KH_2PO_4, 6 g of K_2HPO_4, 0.5 g of sodium citrate, 22 g of glucose monohydrate, 1 g of $MgSO_4 \cdot 7H_2O$, 0.11 mM kanamycin, 1 ml of a decanol (Asahi Denka Kogyo K.K., Japan), and 1 ml of trace element solution. The pH was kept at 7.0 by titration with 25% ammonia; the temperature was 25 °C and aeration was 5 liters/min. The partial pressure of dissolved O_2 was controlled to 30% by increasing agitation from 300 to 1000 rpm during batch phase and regulating the feed of 60% (w/v) glucose during fed batch phase. Product formation was induced at an A_{600} of 50 by adding 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After fermentation the cells were removed by centrifugation at 8000 × *g* for 40 min at 4 °C. The clarified medium was either analyzed and purified directly or stored at −70 °C.

Purification Procedures—DNA present in the clarified medium was removed using precipitation with 0.19% polyethylenimine and 0.2 M NaCl for 30 min (20). After centrifugation as above, the supernatant was collected, and the NaCl concentration was adjusted to 0.5 M. This medium was applied to a protein G-Sepharose column (Pharmacia Biotech Inc.) equilibrated with 0.1 M sodium citrate, pH 6.0, containing 0.05% Tween 80. The column was washed with 1.5–2 column volumes of 0.1 M sodium citrate, pH 6.0, 0.05% Tween 80, 7.5 column volumes of 20 mM citric acid, 1 mM EDTA, 200 mM NaCl, 0.05% Tween 80, pH 4.7, and bound protein was eluted with 0.1 M acetic acid and 0.05% Tween 80. The pH of the sample was adjusted to 5.0 using 0.5 M sodium citrate, pH 7.5, diluted to a conductivity of 4.7 millisiemens/cm, applied to an SP-Sepharose HP column (Pharmacia), and equilibrated with 60 mM sodium acetate, pH 5.0, and 0.02% Tween 80. The column was then washed with 7.5 column volumes of equilibration buffer, and the fusion protein was eluted using a linear gradient from 60 to 350 mM sodium acetate over 13 column volumes.

Analytical Procedures—Cell extracts were prepared from 10 ml of freeze-thawed cell suspension containing both growth medium and cells. The samples were sonicated on an ice bed at an amplitude control of 35% for 3 min using a 3-mm probe, pulsing 70% of the time (VCX .600, Sonics & Materials Inc., Danbury, CT). After sonication, the samples were centrifuged at 8000 × *g* for 40 min, and the supernatants were analyzed. Product levels were measured in a sandwich enzyme-linked immunosorbent assay detecting assembled heterodimeric fusion protein. The wells were coated with antibodies directed against murine light chains, and full-length fusion protein was detected using horseradish peroxidase-conjugated antibodies against SEA. Standard curves were obtained for respective variant fusion proteins. After cell separation, DNA levels in the culture broth were measured using a DynaQuant 200 minifluorometer (Hoefer Scientific, San Francisco, CA) as recommended by the supplier.

Reverse phase HPLC was carried out on an AsahiPak ODP-50 column (4 × 250 mm) (Hewlett-Packard, Palo Alto, CA) using a linear gradient from 30 to 40% acetonitrile in 0.1% trifluoroacetic acid for 40 min and a flow rate of 1 ml/min at 60 °C. Absorbance was measured at 215 nm using a diode array detector (Hewlett-Packard). SDS-polyacrylamide gel electrophoresis was performed on precast Tris/glycine gels (NOVEX, San Diego, CA) containing 12% polyacrylamide. The products were analyzed as both reduced and non-reduced samples using the methods recommended by the supplier. Isoelectric focusing was performed on precast gels (Servalyt® Precotes®, Serva, Heidelberg, Federal Republic of Germany) with a pH working range from 3 to 10 using the methods recommended by the supplier. Mass spectrometry was carried out on a MALDI-TOF MS (Hewlett-Packard), and amino acid analysis was performed using a Beckman 6300 essentially as described (19).

Cytotoxicity Assay—Cytotoxicity was measured in a ^{51}Cr release assay after 4 h using the 5T4 antigen-positive Colo205 cultured in complete tissue culture medium (21) as target cells and human SEA-reactive T cell lines (12) at an effector to target ratio of 30:1. ^{51}Cr-labeled target cells were used in the assay at 2500 cells/200 μl of tissue culture medium in V-bottomed microtiter wells. 5T4Fab-SEch fusion proteins were added at various concentrations, and ^{51}Cr release was measured in a γ-counter. Specific cytotoxicity was calculated as 100 × [(cpm experimental release − cpm background release)/(cpm maximal release − cpm background release)].

Determination of Antigen Binding Characteristics—The human cancer cell lines Calu-1 (ATCC HTB 54) and ME-180 (ATCC HTB 33), both expressing high levels of 5T4 antigen as demonstrated by fluorescence-activated cell sorter staining, were cultivated in tissue culture medium as above. Adhered cells were detached from the flasks using non-enzymatic cell dissociation solution (Sigma), washed twice in a CO_2 independent medium without L-glutamine (Life Technologies, Inc.) containing 10% fetal calf serum, and finally suspended in that medium at a density of 6 × 10^5 cells/ml.

The 5T4Fab-SEch was radiolabeled with the lactoperoxidase technique using Enzymobeads (DuPont NEN). The reaction was stopped with 0.05% NaN_3, and the labeled protein was desalted by gel filtration (PD-10, Pharmacia) using culture medium as elution buffer. Conditions were chosen to obtain a ratio of iodine to protein of ≤2:1.

In a direct binding assay, 3 × 10^4 cells in 50 μl of solution were mixed with 50 μl of serially diluted radioiodinated fusion protein in a conical polypropylene tube in triplicate and incubated for 2 h at room temperature with intermittent mixing. Each tube was washed three times using 3 ml of phosphate-buffered saline containing 1% fetal calf serum, which was removed by centrifugation for 5 min at 600 × *g*. After the final wash, cell-bound radioactivity was determined in a γ-counter. The apparent dissociation constant and number of binding sites at saturation were calculated (22) after subtraction of nonspecific binding (*i.e.* binding after incubation in the absence of cells). This method was modified to an inhibition assay. Here serially diluted fusion proteins competed with wild-type ^{125}I-5T4Fab-SEch at a concentration corresponding to the K_d value determined in the direct assay. The concentration yielding half-maximum inhibition, IC_{50}, was determined after linear regression of log-logit transformed binding data, and the relative affinity index was determined as the ratio between the IC_{50} values of competitor and wild-type 5T4Fab-SEch.

Computer Modeling of the 5T4Fab Variable Region—The individual 5T4 V_H and V_L domains were built by homology modeling to known structures using the COMPOSER module in SYBYL 6.22 (Molecular modeling program SYBYL 6.22, Tripos Associates, St Louis, MO). A family of structurally homologous molecules with sequence identities of at least 60% to the modeled chains were used as templates. For the heavy chain, 7 immunoglobulin fragments were selected, while for the light chain, 22 fragments were used. The structurally conserved regions were built by averaging the template structures according to the COMPOSER algorithm. The remaining LOOP regions were built using template loop fragments found among Fab fragments in the protein structure data base of COMPOSER. The individual V_H and V_L domains were docked to each other to form the Fv fragments. A structural alignment between the individual V_H and V_L chains and the crystallographic structure of a murine Fab, entry 1MCP in the Protein Data Bank (23), was made using the ALIGN procedure of the ICM program (24). Finally, hydrogen atoms were added, and the structure was refined by a regularization procedure in the ICM algorithm (24).

RESULTS

Cloning and Expression of Recombinant 5T4Fab-SEch Constructs—The variable regions of the antibody 5T4 were cloned using PCR and introduced into an expression vector (12) coding for a Fab product with a superantigen linked to the C terminus of the heavy chain. The plasmid was transformed into an *ompT* strain of *E. coli*, UL635, and expression of the recombinant product was induced with IPTG. The product was secreted as

TABLE I

Biochemical and immunological characterization of the different fusion proteins studied

Using an enzyme-linked immunosorbent assay method the level in the growth medium and total yield in a sonicated mixture of growth medium and bacteria were determined. The IC_{50} values were obtained from affinity measurements to 5T4 antigen-positive cells, and the biological activity was determined using a cytotoxicity assay. The variants of the 5T4 heavy chain contain the following substitutions: H41P, S44G, I69T, and V113G (mutant 1 (m1)) and H41P, S44G, and V113G (mutant 2 (m2)).

Variant	Light chain replacements								Heavy chain	Yield		IC_{50}	Activity
	F10S	T45K	I63S	Y67S	F73L	T77S	L78V	L83A		Medium	Total		
										mg/liter		*nM*	%
wt									5T4	39	48	1.5	100
V1									C215	39	59	ND[a]	0
V2	C215-κ								5T4	224	297	1000	0
V3	X[b]								5T4	92	126	1.2	100
V4			X	X					5T4	39	52		
V5	X		X	X					5T4	93	136	2.9	100
V6					X				5T4	39	44		
V7						X			5T4	57	86		
V8							X		5T4	53	77		
V9								X	5T4	53	86	1.6	100
V10	X	X	X	X					5T4	214	250	2.6	100
V11	X	X			X	X	X		5T4	586	701		
V12	X		X	X	X	X	X		5T4	512	470	2.0	100
V13	X	X	X	X	X	X	X		5T4	578	560	1.4	100
V14	X	X	X	X	X	X			5T4 (m1)	288	730	1.9	100
V15	C215κ								5T4 (m2)	250	560		
V16			X	X	C215-(1–23)-5T4				5T4	110	124	5.4	100

[a] ND, not detected.

[b] X, substitution introduced in the respective variant.

two separate polypeptide chains that assembled to a heterodimeric product in the *E. coli* periplasm. During fermentation, a significant amount of the two-chain product is excreted to the growth medium and usually connected by a significant cell lysis. Normally the levels of Fab superantigen products range from 100 to 400 mg/liter in the growth medium (data not shown). However, for 5T4Fab-SEch the production level was around 40 mg/liter in the growth medium and less than 10 mg/liter in the periplasm, as determined with enzyme-linked immunosorbent assay (Table I). To increase the yield, several parameters in the fermentation procedure were varied such as time point and level of induction, temperature, and medium composition, but no further improvement in production level was achieved.

Construction and Investigation of Hybrid Molecules between 5T4-SEch and C215Fab-SEch—To determine whether one of the two polypeptide chains dominated the production problem, hybrid molecules of 5T4Fab-SEch and C215Fab-SEch were made. C215 is a murine antibody recognizing a colon cancer epitope (25), and fusion proteins between C215Fab and SEA-based superantigens are normally secreted at levels up to 400 mg/liter in *E. coli*. Fermentation of variant V1 with the 5T4 light and C215 heavy chain yielded 39 mg/liter product in the growth medium, while variant V2 with C215 light and 5T4 heavy chain yielded 224 mg/liter (Table I). For V1 and V2, most of the product was found in the growth medium instead of the periplasm (Table I). Hence, replacing the heavy chain of 5T4 did not affect the low production level, while replacement of the light chain resulted in a more than 5-fold increase.

One further observation was made during fermentation. For the 5T4 wild-type construct, as well as variant V2 that contained the C215 light chain, the A_{600} started to decline and the cell viability decreased more than 10-fold within a few hours after induction (Fig. 3), followed by an increased DNA level in the growth medium. The variant V1 that contained the C215 heavy chain behaved differently. The amount of viable *E. coli* cells was more than 10-fold higher for V1 than for V2 with 5T4 heavy chain when fermentation was terminated. In addition, the final cell density was much higher (Fig. 3).

The fusion proteins were purified using protein G affinity and then ion exchange chromatography to remove degraded forms. The purified products were analyzed with SDS-polyacrylamide gel electrophoresis, reverse phase HPLC, mass spectrometry, isoelectric focusing, and amino acid analysis. The latter technique was also used to determine protein concentrations. All of these assays indicated that the main product contained and constituted 85–95% of the expected characteristics. However, both products showed a strongly reduced affinity for the 5T4 antigen (16), and only variant V2 with the 5T4 heavy chain had measurable affinity. The IC_{50} value was lowered approximately 1000-fold, and the products were at least 1000-fold less potent in cytotoxic activity (Table I).

Three important conclusions could be made from these data. The low yield of product was mainly associated with the 5T4 light chain; the high cell lysis during fermentation was primarily associated with the 5T4 heavy chain, and both chains contain residues important for binding to the 5T4 antigen.

Molecular Modeling of 5T4 Fv—To explain the low production level of 5T4, a model was built. Here information regarding exposed hydrophobic residues, structural identification of the complementarity-determining regions, CDRs, and insights into the structural environment of the residues described below was obtained. The high sequence identity of more than 60% to a relatively large number of template structures ensured that the overall accuracy of the model was good. The most uncertain regions are those modeled as LOOP regions, *e.g.* either not structurally conserved within the family of template structures or not highly homologous to them. However, for these LOOP regions such templates, which fitted well to the structurally conserved regions, were found among other immunoglobulin structures. Of the residues investigated only Tyr-67 in the light chain and Val-113 in the heavy chain were situated in the LOOP regions. Therefore, the model most likely correctly predicts whether the residues studied were exposed or buried and whether a certain residue is needed to support the CDR loop structure.

Engineering of the 5T4Fab-SEch Construct—Based on the finding that V_L replacement in 5T4Fab efficiently increased the production level but affected the binding properties, the molecule was modified to identify residues that hampered high level production. Hydrophobic residues, suggested to be on the Fab surface by computer modeling (Fig. 2), were replaced by serine

COMPARISON OF THE LIGHT CHAIN VARIABLE DOMAINS

```
          1              XXXX          XXXXX XXXXXXXX        XXXXX       XXXXX
C215      DIVMTQSPSS LTVTAGEKVT MNCKSSQSLL NSRNQKNYLT WYQQKPGQPP KLLIYWASTR
5T4       SIVMTQTPTF LLVSAGDRVT ITCKASQSVS ND......VA WYQQKPGQSP TLLISYTSSR
          1         *                                             *
                    S                                             K

          XX  XXX        XXX          XXXXX      XXXXXX XXX         XXX
C215      ESGVPDRFTG SGSGTDFTLT ISSVQAEDLA VYYCQNDYVY PLTFGAGTKL ELK
5T4       YAGVPDRFIG SGYGTDFTFT ISTLQAEDLA VYFCQQDYNS PPTFGGGTKL EIK
         55         *    *     *    **    *
                    S    S     L    SV    A
```

COMPARISON OF THE HEAVY CHAIN VARIABLE DOMAINS

```
          XX        X XXXXX           XXXXX XX          XXX           XXXX   X
C215      QVQLQQPGAE LVRPGASVKL SCKASGYTFT NYWINWVKQR PGQGLEWIGN IYPSYIYTNY
5T4       EVQLQQSGPD LVKPGASVKI SCKASGYSFT GYYMHWVKQS HGKSLEWIGR INPNNGVTLY
          1                                           *  *
                                                      P  G

 XXXXX         XXX          XXXXXXXX X         X XXXXXXXXXX XXX         xxXX
 NQEFKDKVTL TVDESSSTAY MQLSSPTSED SAVYYCTRSP YGYDE...YG LDYWGQGTSV TVSS
 NQKFKDKAIL TVDKSSTTAY MELRSLTSED SAVYYCARST MITN....YV MDYWGQVTSV TVSS
61        *                                                *
          T                                                G
```

FIG. 1. **Amino acid sequence of the Fv part of the antibodies 5T4 and C215 showing the substitutions examined.** The one-letter abbreviations are used for the amino acid residues. The CDRs (15) are indicated in *bold*, and the approximate positions of the loops are indicated by the letter *X*.

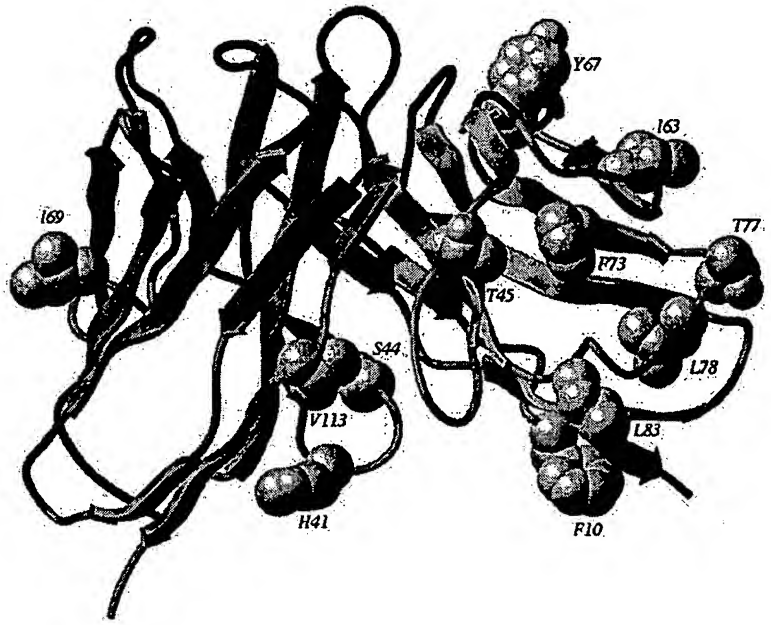

FIG. 2. **Ribbon representation of the 5T4 Fv model.** Substituted amino acid residues are indicated as space-filling models, and the CDRs are indicated by *black ribbons*.

residues. Selected residues differing from the equivalents in more readily produced Fab fragments such as C215 were exchanged for the latter (Fig. 1). To minimize putative effects in affinity and specificity, residues in the CDRs were not altered. The C_H1 and C_L regions were identical in all antibodies studied. The chosen substitutions were Phe-10 → Ser, Thr-45 → Lys, Ile-63 → Ser, Tyr-67 → Ser, Phe-73 → Leu, Thr-77 → Ser, Leu-78 → Val, and Leu-83 → Ala in the light chain. In addition, to identify heavy chain residues that could affect the yield or cell lysis as suggested by the hybrid studies, the substitutions His-41 → Pro, Ser-44 → Gly, Ile-69 → Thr, and Val-113 → Gly were investigated. The positions of these residues and a sequence alignment between the Fv regions of 5T4 and C215 are shown in Figs. 1 and 2. In the model, Phe-10, Thr-45, Ile-63, and Thr-77 in the light chain are exposed side-chain residues. Consequently, the replacements Phe-10 → Ser, Ile-63 → Ser, and to a lower degree Thr-77 → Ser should make the product less hydrophobic. The substitutions Phe-73 → Leu and Leu-78 → Val were made in the completely buried hydrophobic core of the light chain. The light chain residue Tyr-67 is in a loop close to the CDRs. Replacing this residue may therefore change the binding properties of the molecules. The heavy chain substitutions His-41 → Pro and Ser-44 → Gly involved exposed side chains positioned at the N and C terminus, respectively, of a sharp turn connecting two framework β strands. Both proline and glycine residues are important in stabilizing sharp turns in proteins. The substitutions Leu-83 → Ala in the light chain and especially Val-113 → Gly in the heavy chain may affect the interactions with the constant domains. Although not modeled, these residues are in the domain-domain interface of structural homologues. Finally, to find out if other residues in framework 1 affected the yields, a variant of 5T4 containing the 23 N-terminal residues of the C215 light chain instead of the wild-type ones, was constructed. The effects of the different substitutions were investigated as single or combined amino acid replacements. In a reverse phase HPLC system (Fig. 4), the variant chains of 5T4 are much more hydrophilic than the wild-type chains.

Impact of Engineering on Production Levels—The hybrid variants of 5T4 and C215 suggest a replacement of critical light chain residues in 5T4 to obtain a higher production level. Indeed, enzyme-linked immunosorbent assay measurements

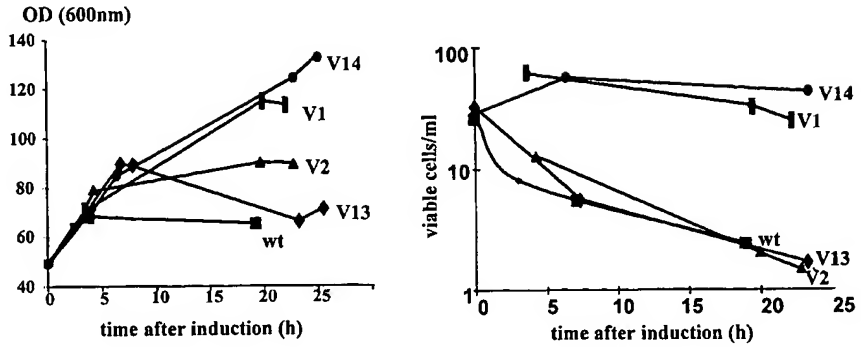

FIG. 3. **Growth curves and cell viability measured during fermentation of the wild-type and variant 5T4Fab-SEch constructs.** The variants shown are V1, V2, V13, and V14 (Table I). Variants V1 and V14, containing the C215 or a mutant heavy chain, differ markedly from the wild-type construct since the A_{600} (*OD*) reaches a much higher level, and the number of viable cells ($\times 10^9$) is not markedly decreased.

after fermentation showed that individual substitutions had substantial impact on the yields. Notably, a single substitution Phe-10 → Ser, variant V3, increased the level from 39 to 92 mg/liter in the growth medium (Table I). Further substitutions increased the production levels continuously and by introducing five or seven point mutations in the light chain, variants V11–V13, the growth medium levels exceeded 500 mg/liter. For these variants, the V_L moiety may no longer be the limiting component. Phe-10 → Ser was the most important replacement, followed by similar and almost additive effects from Thr-45 → Lys, Thr-77 → Ser, and Leu-78 → Val. Furthermore, Leu-83 → Ala also enhanced the yield but was not studied in combination with the others. Except for Phe-10 → Ser, replacing the complete framework 1 did not drastically alter the level as seen in variants V5 and V16.

Significant cell lysis was observed during cultivation of wild-type 5T4Fab-SEch and the most product was found in the growth medium. A few hours after induction with IPTG, the cell growth was markedly affected, and the cell mass in the fermenter started to decline, as determined by A_{600} (Fig. 3). In addition, the number of viable cells decreased more than 10-fold within 10–15 h (Fig. 3). These characteristics were not fundamentally changed by any of the light chain alterations in variants V3 to V13. However, substitutions in the heavy chain altered the properties markedly. The cell mass continued to increase throughout the fermentation to an A_{600} of almost 150 (Fig. 3) with repressed cell lysis. As a consequence, the most product was found in the periplasm. In variant V14 with seven light chain and four heavy chain substitutions, the level was 288 mg/liter in the growth medium and almost 450 mg/liter in the periplasm (Table I). Subsequently, combined with a suitable light chain, the heavy chain replacements increased the total level of fusion protein to 30%. The DNA levels in the growth medium, reflecting the cell lysis, showed that variant V13 contained more than 1 g of DNA/liter, while V14 contained less than 0.2 g. A hybrid molecule, V15, with C215 light chain and 5T4 heavy chain with the replacements His-41 → Pro, Ser-44 → Gly, and Val-113 → Gly, was also investigated. This molecule gave approximately the same yield of product in the growth medium, 250 mg/liter, as hybrid V2. However, similarly to variant V14, cell lysis was less pronounced with this heavy chain, indicating that the substitution Ile-69 → Thr was less important for increased cell viability.

Thus, replacing a few residues in the 5T4 light chain increased the yield almost 15-fold and was further augmented by heavy chain substitutions. The heavy chain replacements altered the phenotype of the *E. coli* cells during fermentation, and less lysis was observed. Subsequently, most of the product was found in the periplasm instead of the growth medium.

Analysis of the Mutated Forms of 5T4—Similar to the 5T4 and C215 hybrids, the variants of 5T4Fab-SEch were purified, and biochemical analyses showed approximately 85–95% of the main component (Fig. 4) with expected characteristics. The minor products seen on reverse phase HPLC are isomers of the light or heavy chains that are not separated from the wild-type chains on SDS-polyacrylamide gel electrophoresis.

To investigate whether the replacements affected biological properties, the different products were analyzed for binding to the 5T4 antigen, and since the constructs were aimed for immunotherapy, a functional *in vitro* assay was also performed. None of the substitutions seemed to have a significant effect on cytotoxic activity (Table I), but replacement of Ile-63 and Tyr-67 with serine residues as in variants V4, V5, and V10 resulted in a reduced affinity for the antigen by approximately 50% (Fig. 5 and Table I). Surprisingly, this effect was reversed by the light chain substitutions Phe-73 → Leu, Thr-77 → Ser, and Leu-78 → Val in variants V12 and V13. The variant V16 containing the 23 N-terminal residues from C215 combined with the substitutions Ile-63 → Ser and Tyr-67 → Ser had an affinity of approximately 30% compared with the wild-type 5T4Fab-SEch (Fig. 5 and Table I). This indicated that unknown residues in framework 1 of 5T4 stabilized the antigen binding site, and if replaced by the equivalents from C215, the binding properties were affected. These effects were not studied further.

In conclusion, none of the replacements resulted in a dramatic alteration in either affinity or cytotoxic activity of the 5T4Fab-SEch molecule. However, some of the substitutions slightly changed the binding properties.

DISCUSSION

It was recently demonstrated that particular amino acid residues in the CDRs of recombinant antibodies can influence the level of secreted product in *E. coli* (26, 27). Here that finding was extended showing that Fv framework substitutions significantly enhanced the yield of a secreted Fab-fusion protein in *E. coli*. Two approaches were used to design variants of the antibody 5T4. Hydrophobic residues, likely to be on the framework surface according to molecular modeling, were replaced with Ser or Ala, and a few less frequent residues were replaced by those of an antibody that can be produced at relatively high yields in *E. coli* (Fig. 1). To minimize the risk of changing the binding properties, CDR engineering was not performed. Using only five light chain substitutions, the product level in the growth medium increased approximately 15 times without significantly modifying the affinity for the antigen. This level was higher than for the model antibody C215. The high producing variants V11–V13 all reach a level above 500 mg/liter, and here the V_L part may not be the limiting component. The hydrophobic light chain residue Phe-10, which is totally exposed in the model (Fig. 2), was very limiting for high level production. In variant V3, Phe-10 was replaced with Ser, which resulted in a 2.5-fold increase in the production level. In addition, the substitutions Thr-45 → Lys, Thr-77 →

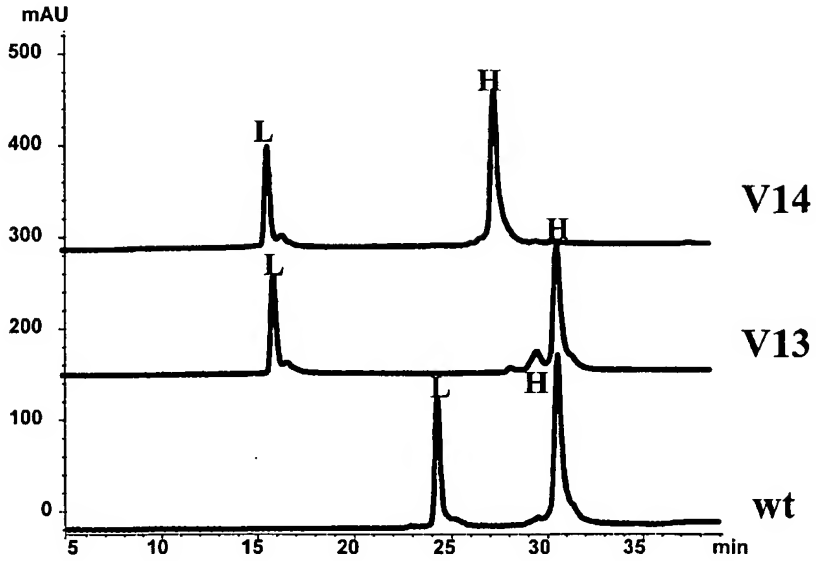

FIG. 4. **Reverse phase HPLC analysis of purified wild-type 5T4Fab-SEch, variant V13 with seven substitutions in the light chain, and V14 with seven substitutions in the light chain and four in the heavy chain.** The analytical conditions are described under "Experimental Procedures." Since the polypeptide chains are not covalently attached to each other, they are detected individually, and it is estimated that each product has a homogeneity of 85–95%. *L*, light chains; *H*, heavy chains; and *wt*, wild-type. In this assay, the variant chains are significantly less hydrophobic than wild type.

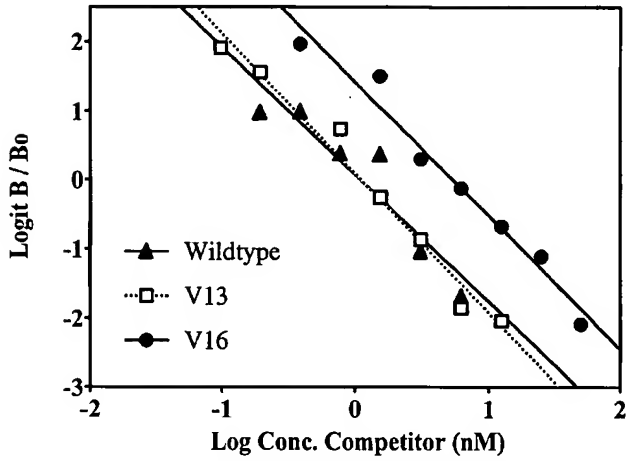

FIG. 5. **Determination of the affinities for the 5T4 antigen in one representative experiment for wild-type 5T4Fab-SEch and its variants V13 and V16.** The affinities were determined on Calu-1 cells as described under "Experimental Procedures." Variant V16 has a relative affinity of approximately 20% compared with wild-type and variant V13 (Table I).

Ser, Leu-78 → Val, and Leu-83 → Ala increased the yield especially when Phe-10 was replaced as observed with the variants V5 and V7–V13 (Table I).

While the light chain substitutions had a tremendous impact on the final yield, the heavy chain replacements primarily affected product localization (Fig. 3, Table I). Thereby, a tool that enables targeting of recombinant antibody fragments to either the periplasm or the growth medium might have been identified. Whether it is optimal to obtain a recombinant product in the periplasm or growth medium can be questioned, but for downstream processing there are definite advantages to recovering a product from the growth medium. In accordance with this study, a recent investigation of heavy chain loop substitutions show that residues controlling production level and periplasmic leakiness may differ (26). A Pro as residue 40 led to a higher leakiness compared with Ala, while the substitutions Ser-61 → Ala and Ala-62 → Asp (using the 5T4 positions) lead to higher production levels. Notably, Pro-40 resided in the corresponding turn that appeared to be important for periplasmic leakiness in this study. By comparing previously reported yields with our results, it seems possible that the residues studied here generally determine the production level for secreted antibody fragments. For instance, one antibody reported to be produced very poorly in *E. coli* contains a Phe at position 10 (27). Also, the humanized Fab secreted in approximately 1 g/liter (7) contains most of the optimal residues like Ser-10, Lys-45, and Ser-77 as well as those found important by Knappik and Plückthun (26). The heavy chain substitution Ile-69 → Thr seems to have less impact on product levels and localization (Table I) (26).

There are several possible explanations for the drastic differences observed. For instance, compared with the engineered variants, wild-type 5T4Fab may have poor folding properties, lower solubility of the unpaired chains, a higher tendency for aggregation, a higher formation rate of unproductive light chain dimers (28), or a lower stability toward proteolysis (29). Also the wild-type mRNA could have a low stability, or less likely there may be problems with the translocation initiation process (30). The periplasmic folding process has been suggested to be the major limitation for secretion of recombinant antibodies in *E. coli* (31, 32). Furthermore, replacing residues identified as limiting indeed improved *in vitro* refolding of reduced and denatured Fv molecules (26), and proline isomerization was rate-limiting for that folding process (33). It is therefore likely that at least some of the substitutions in 5T4 caused an effect that facilitated proper folding. Replacing hydrophobic residues on protein surfaces with more hydrophilic ones has yielded products with suppressed tendencies for aggregation or dimer formation during production (34). Therefore, the variant light chains of 5T4, which are more hydrophilic than the wild-type chain, are probably less prone to aggregate. Preliminary analyses on the amount of light chain dimers indicate that in no case does the level exceed that of Fab (data not shown). Recently, a folding model for recombinant antibodies in *E. coli* was suggested (33). Here, the light chain acts as a folding template for the heavy chain that would otherwise aggregate. Our data do not contrast that model. Thus, the final yield was probably determined by the folding and aggregate-forming properties of the light chain and the time needed for the heavy chain to find its partner chain before precipitation, which may induce stress to the host cell.

None of the substitutions in 5T4 resulted in a drastic change in affinity for the antigen. According to the model (Fig. 2) only Tyr-67 is positioned close to the CDRs. Combining the substitutions Ile-63 → Ser and Tyr-67 → Ser or replacing the light

chain framework 1 resulted in a decrease in affinity by approximately 50%, but additional substitutions reversed this effect (Fig. 5 and Table I). Similar to previous experiences (35, 36), this shows that particular residues in individual framework regions stabilize the unique conformation of an antibody's antigen binding site. For tumor therapy it is unclear what affinities are optimal, but the repertoire of antibodies generated here, perhaps differing in the k_{on} and k_{off}, may be used to investigate these issues.

The mechanisms inducing leakage of proteins from periplasm to growth medium are not well known (37, 38). The data presented here and elsewhere (26, 39) show that small variations in the composition of a secreted product can induce a large difference in stress for the host cell, which leads to lysis during fermentation. Cell lysis was certainly one important reason for the high amounts of product found in the growth medium in this study, but apparently leakiness that was not directly coupled to lysis was observed in two different ways. First, despite great similarities between variants V1, V14, and V15 regarding viable cells, etc. during fermentation (Fig. 3), the ratios of product found in the growth medium and periplasm differ (Table I). Second, even though replacing heavy chain residues resulted in a higher viability of the *E. coli* cells and subsequently less lysis, at least 40% of the product was still found in the growth medium. It is less likely that all of this product was released by cell lysis since after fermentation of variant V14 the DNA level in the growth medium was less than 20% compared with V13. Also, a few other recombinant products tend to be found primarily in the growth medium after secretion without any connected cell lysis (40, 41). Consequently, there must be a complex relationship between how individual residues affect cell lysis and periplasmic leakage. Further studies with this system may explain some of the molecular mechanisms behind these events for antibody fragments.

In conclusion, this paper has shown that problems with low production of a secreted antibody fragment may be circumvented by molecular engineering. It may also be feasible to modify the framework so the product can be recovered from either the growth medium or the periplasm. More speculatively, the substitutions identified here in combination with those found by others could constitute a platform for the design of frameworks that are generally suitable for *E. coli* production of recombinant antibodies.

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Determinants of Specificity of a Baculovirus-expressed Antibody Fab Fragment That Binds Selectively to the Activated Form of Integrin $\alpha_{IIb}\beta_3$*

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PAC1 is an IgMκ murine monoclonal antibody that, like the Arg-Gly-Asp-containing ligand fibrinogen, binds to integrin $\alpha_{IIb}\beta_3$ only on activated platelets. The unique binding properties of PAC1 may be determined by its large size, its multivalency, and by variable region sequences, including an Arg-Tyr-Asp at residues 100A-C in H-CDR3. To study the molecular determinants of PAC1 function, baculoviruses containing cloned cDNA for the Fd heavy and κ light chains of PAC1 were used to coinfect Sf9 insect cells. Infected cells secreted a soluble, monovalent, 50-kDa Fab fragment that bound saturably to agonist-stimulated platelets but not to resting cells. Fab binding was inhibited >85% by 10 mM EDTA, 1 mM RGDS, 1 mM fibrinogen $\gamma_{397-411}$, or 12 μM fibrinogen, but not by 1 mM RGES. Compared to PAC1 IgM, a 60-fold higher molar concentration of PAC1 Fab was required for half-maximal binding to platelets or for half-maximal inhibition of fibrinogen binding. PAC1 Fab bound to an activated form of $\alpha_{IIb}\beta_3$ expressed in Chinese hamster ovary cells, but not to the resting form of the receptor in these cells or to $\alpha_v\beta_3$ in human endothelial cells. Conversion of Asp^{100C} to Glu by site-directed mutagenesis rendered the antibody inactive, indicating that the Arg-Tyr-Asp sequence in H-CDR3 is essential for PAC1 recognition of $\alpha_{IIb}\beta_3$. Binding of fibrinogen or PAC1 IgM to platelets induced tyrosine phosphorylation of a 140-kDa platelet protein, but binding of PAC1 Fab did not. These studies demonstrate that the specificity of PAC1 for activated $\alpha_{IIb}\beta_3$ is determined by an integrin recognition sequence within H-CDR3. However, the strength of this binding interaction and the ability of PAC1 to trigger signaling in platelets also depend on antibody valency.

Integrins are transmembrane αβ heterodimers that mediate cell-cell and cell-matrix interactions during normal physiological processes, such as embryonic development, immune responses, or hemostasis, and during pathological processes, such as neoplasia or thrombosis (1–3). Human platelets have provided a useful model system for examining integrin-mediated events in hemostasis and thrombosis (4, 5). Of the five integrins expressed on platelets ($\alpha_{IIb}\beta_3$, $\alpha_v\beta_3$, $\alpha_2\beta_1$, $\alpha_5\beta_1$, and $\alpha_6\beta_1$), $\alpha_{IIb}\beta_3$ is the most abundant, and its interaction with the Arg-Gly-Asp (RGD)-containing adhesive proteins, fibrinogen and von Willebrand factor, is essential for platelet spreading on extracellular matrix and for platelet aggregation (6, 7). The apparent affinity of $\alpha_{IIb}\beta_3$ for these ligands is tightly regulated by the cell, a phenomenon that has been referred to as "inside-out" signaling (8). Resting platelets do not bind fibrinogen with measurable affinity, while thrombin-activated platelets bind approximately 40,000 fibrinogen molecules/cell with an apparent K_d of 300 nM (6, 8). In addition to inside-out signaling, "outside-in" signaling is triggered by fibrinogen binding to $\alpha_{IIb}\beta_3$, which causes clustering of integrin receptors and tyrosine phosphorylation of specific platelet proteins (9, 10).

PAC1 is a murine IgMκ monoclonal antibody that recognizes $\alpha_{IIb}\beta_3$ and mimics the binding characteristics of fibrinogen. For example, while PAC1 fails to bind to resting platelets, it binds to 25,000–30,000 sites per platelet after thrombin stimulation, with an apparent K_d of 5 nM (11). Binding of either PAC1 or fibrinogen is inhibited competitively by the other ligand as well as by synthetic peptides derived from receptor recognition sequences in fibrinogen (*e.g.* RGDF and RGDS from the Aα chain, and $\gamma_{397-411}$ from the COOH terminus of the γ chain (11–13)). The similarities between fibrinogen and PAC1 are underscored by the observation that anti-idiotypic antibodies to PAC1 bind to fibrinogen (14). This mimicry of fibrinogen may be explained, at least in part, by the structure of one of the six hypervariable regions in PAC1, H-CDR3 (R^{95}SPSYYRYDGAGPYYAMDY^{102}; numbering system according to Kabat *et al.* (15)). A synthetic peptide derived from this CDR inhibited both PAC1 and fibrinogen binding to activated platelets. In contrast, a peptide containing the inverted DYR sequence was inactive (16). Molecular modeling studies suggest that the Arg-Tyr-Asp (RYD) within H-CDR3 is situated at the apex of a β-loop (17, 18), much like the orientation of RGD sequences in constrained bioactive peptides known to inhibit fibrinogen binding to $\alpha_{IIb}\beta_3$ (19–21). These studies suggest that the antibody-combining site of PAC1 may share a three-dimensional resemblance to one or more of the integrin recognition domains in fibrinogen.

Two outstanding issues in $\alpha_{IIb}\beta_3$ biology include: 1) the molecular basis of affinity modulation of the receptor, and 2) the structural features of a ligand that determine its specificity for a particular integrin. With regard to affinity modulation, although small RGD peptides can bind to resting platelets, the access of larger ligands, such as the 330-kDa fibrinogen molecule or the 1000-kDa PAC1 molecule, might well be limited by their large size. One way to address this issue would be to perform platelet binding studies with a smaller functional unit of PAC1, such as a Fab fragment. With regard to the issue of integrin specificity, it is known that fibrinogen, von Willebrand factor, and vitronectin can recognize both $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$ (22). In contrast, certain snake venom disintegrins as well as syn-

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thetic RGD peptides and peptidomimetics appear to bind selectively to $\alpha_{IIb}\beta_3$ (23–25). The molecular basis for this selectivity is not fully understood. In this context, it is not known whether PAC1 can bind to $\alpha_v\beta_3$.

The present studies were carried out using recombinant Fab fragments of PAC1 expressed by baculovirus-infected insect cells to study the roles of PAC1 size, valency, and sequence as determinants of the unique binding characteristics of this anti-integrin antibody. Although baculovirus-directed expression of Fab fragments has not yet been reported, this system has been used successfully to express IgG antibodies and single-chain Fv fragments in amounts suitable for biochemical analyses (26–29). The present studies show that insect cells can secrete relatively large amounts of monovalent PAC1 Fab, which binds to $\alpha_{IIb}\beta_3$ on platelets but not to $\alpha_v\beta_3$ on human endothelial cells. Furthermore, despite its 20-fold smaller molecular mass relative to PAC1 IgM, PAC1 Fab binds only to an activated form of $\alpha_{IIb}\beta_3$. Finally, site-directed mutagenesis of PAC1 indicates that integrin specificity is directly dependent on the RYD sequence within hypervariable region, H-CDR3.

MATERIALS AND METHODS

Cell Culture—Monolayer or suspension cultures of Sf9 cells from *Spodoptera frugiperda* were adapted to and maintained at 27 °C in Sf-900 II serum-free medium (Life Technologies, Inc.) supplemented with fungizone and gentamycin (30, 31). CHO[1] cell lines that express recombinant forms of human $\alpha_{IIb}\beta_3$ were constructed and maintained as described (32, 33). The A5 CHO cell line expresses wild-type $\alpha_{IIb}\beta_3$, while the $\alpha_L\Delta$ cell line expresses full-length β_3 along with an α subunit composed of the extracellular and transmembrane domains of α_{IIb} and the cytoplasmic domain of α_L in which the membrane-proximal VGFFK sequence has been deleted.

Construction of Baculovirus Transfer Vectors—The method of Muller and Rajewsky (34) was used to obtain an $IgG_1\kappa$ class-switch variant of PAC1, which was kindly provided by Paul Hering, F. Hoffman-La Roche Ltd., Basel, Switzerland. This variant, which continued to produce both IgG and IgM protein despite multiple rounds of cloning, was used to obtain PAC1 mRNA for construction of baculovirus transfer vectors. PAC1 γ_1Fd (V_H-γ_1CH_1) and κ cDNA were amplified by reverse transcriptase-PCR from total cellular RNA (35). The primers for the heavy chain reaction were 5′-GGCGGTGATCAGCAGGTGCAGCTGAAG-CAG-3′ and 5′- GGCGGACTAGTTTACTAACAATCCCTGGGCACAAT-3′; the primers for the light chain (kappa) reaction were 5′-GGCATG-GATCCAGATGTTTTGATGACCCAAC-3′ and 5′-GGCGGTCTAGA TTACTAACACTCATTCCTGTTGAA-3′. The heavy chain product was digested with *BclI* and *SpeI*, and the light chain with *BamHI* and *SpeI*. These digests were then individually force-cloned into the pVT-Bac baculovirus transfer vector that had been cut with *BamHI* and *NheI* (36); to produce two separate transfer vectors, pVT-Bac/PAC1 Fd and pVT-Bac/PAC1 κ. In each case, the immunoglobulin fragment was fused in-frame to the 3′ end of the signal peptide for honeybee mellitin, immediately downstream of the strong polyhedrin promoter.

To facilitate purification of PAC1 Fab secreted by Sf9 cells, an oligohistidine tail $(His)_6$ was introduced at the 3′ end of the PAC1 heavy chain cDNA, using pVT-Bac/PAC1 Fd as a template and 5′-GGCATCT-GCAGTCTGACCTCTACACTCTGAGCAGC-3′ and 5′-GGCGGAAT-TCTTAGTGATGGTGATGGTGATGACAATCCCTGGGCACAAT-3′ as the PCR primers. The PCR product was digested with *PstI* and *EcoRI* and subcloned into pVT-Bac/PAC1 Fd which had been cut with *PstI* and *EcoRI*. This resulted in the final heavy chain transfer vector, pVT-Bac/PAC1 Fd_{His}, which is illustrated in Fig. 1, along with pVT-Bac/PAC1 κ.

Splice overlap extension PCR was used to construct PAC1 Fab containing an Asp → Glu substitution at amino acid residue 100C in H-CDR3 (15, 37). This mutation was introduced using the primer 5′-ATAGTAAGGACCCGCCCCTTCGTACCTATAGTAGGAG-3′, and the PCR product was cloned into the pVT-Bac vector as described above. The complete sequences of all PCR-generated products in this study were verified to be correct by dideoxynucleotide sequencing (Sequenase version 2.0 kit; U. S. Biochemical Corp.).

Transfection and Isolation of Recombinant Baculoviruses—To obtain recombinant baculoviruses, cotransfection of Sf9 cells was performed with 2 μg of either the pVT-Bac/PAC1 Fd_{His} or the pVT-Bac/PAC1 κ plasmid and 0.5 μg of linearized AcRP23 LacZ Baculovirus DNA or BaculoGold Baculovirus DNA (Pharmingen, San Diego, CA), according to the manufacturer's protocols. Single clones were isolated by plaque assay. Recombination was verified by PCR, and recombinant viruses were amplified and titered by repeat plaque assay (38).

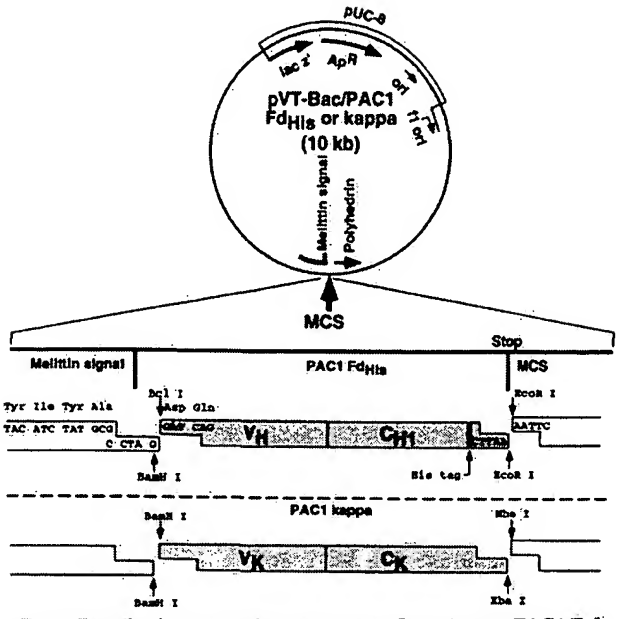

FIG. 1. **Baculovirus transfer vectors used to express PAC1 Fab.** The pVT-Bac/PAC1 Fd_{His} vector was used to clone a recombinant baculovirus for expression of the PAC1 γ_1Fd chain (V_H-γ_1CH_1), and the pVT-Bac/PAC1 κ vector was used for expression of PAC1 κ. Heterodimers of these chains produce PAC1 Fab.

Expression, Purification, and Analysis of Recombinant PAC1 Fab—Small-scale suspension or monolayer cultures of 10–50 ml of Sf9 cells in *Sf900 II serum-free medium* were infected with recombinant baculoviruses at a multiplicity of infection of 3. At 24-h intervals up to 96 h, cell aliquots were pelleted, resuspended, and sonicated in buffer containing 20 mM Hepes, pH 8.0, 2 mM $MgCl_2$, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, and 5 μg/ml leupeptin (39). Sonicates were centrifuged at 10,000 × *g* for 30 min at 4 °C to obtain the soluble, "cytoplasmic" fraction for analysis of PAC1 expression.

Larger scale production of secreted PAC1 Fab was accomplished by co-infecting a 500-ml suspension culture of Sf9 cells in a spinner flask (2×10^6 cells/ml) with recombinant PAC1 Fd_{His} and PAC1 κ baculoviruses at a multiplicity of infection of 3. After 72 h, the cell culture supernatant was collected, supplemented with 0.1 mM phenylmethylsulfonyl fluoride and 5 μg/ml aprotinin and leupeptin, and then dialyzed extensively at 4 °C against phosphate-buffered saline, pH 7.4. This dialysis step was necessary to remove substances from the serum-free media that prevented retention of PAC1 Fab on the metal chelate affinity column. The dialyzed supernatant was incubated with gentle rocking overnight at 4 °C with 2 ml of Ni-NTA resin (Quiagen, Inc., Chatsworth, CA). The mixture was then poured into a 10 × 1.5-cm column, washed with 100 ml of phosphate-buffered saline to remove unbound proteins, washed again with 100 ml of 10 mM imidazole (Sigma) to remove nonspecifically bound proteins, and then eluted with 250 mM imidazole to specifically elute PAC1 Fab. One-half-ml aliquots were monitored for protein at A_{280} and aliquots containing detectable protein were pooled for further analysis. Protein concentration was determined by the BCA method (Pierce, Rockford, IL).

Protein samples were analyzed by 10% SDS-polyacrylamide gel electrophoresis (40). Gels were either stained for protein with Coomassie Brilliant Blue or transferred to nitrocellulose for immunoblotting (14, 41). Blotting reactions were carried out with Cg2–236 hybridoma tissue culture supernatant (containing a monoclonal antibody specific for mouse γ_1CH1 (42); a gift from Fred Karush University of Pennsylvania) and/or with an affinity-purified rabbit polyclonal antibody against mouse Ig (H+L) (Zymed Laboratories, S. San Francisco, CA). Binding of each of these antibodies was detected with an appropriate second antibody coupled to horseradish peroxidase using enhanced chemiluminescence (Amersham Corp.). The apparent size of PAC1 Fab in aqueous

[1] The abbreviations used are: CHO, Chinese hamster ovary; PCR, polymerase chain reaction; FITC, fluorescein isothiocyanate.

solution was estimated by size exclusion chromatography on a Sephadex G-200 column using the following proteins as standards: hen egg lysozyme (14.4 kDa), bovine serum albumin (66.2 kDa), and an unrelated monoclonal IgG antibody (PAC4, 150 kDa).

Flow Cytometric Analysis—Binding of recombinant PAC1 Fab to fresh gel-filtered human platelets was analyzed by flow cytometry as described (43). The final incubation buffer contained 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 0.1 mM $CaCl_2$, 5.6 mM glucose, 1 mg/ml bovine serum albumin, 3.3 mM NaH_2PO_4, and 20 mM HEPES, pH 7.4 (11). Reactions were started by adding PAC1 Fab and various agonists and/or inhibitors to 50 μl of 5×10^5 platelets. The agonists included phorbol myristate acetate, L-epinephrine, ADP (all from Sigma), or bovine thrombin (Calbiochem). Potential inhibitors included prostaglandin (Sigma), EDTA, fibrinogen, or peptides such as RGDS, RGES, or fibrinogen $\gamma_{397-411}$ (Peninsula Laboratories, Inc., Belmont, CA) (14). After a 15-min incubation at room temperature, fluorescein isothiocyanate (FITC)-labeled rabbit anti-mouse Ig (Zymed; 143 μg/ml) was added for an additional 15 min, and the samples were analyzed in a Becton-Dickinson FACStar flow cytometer. The FITC-labeled second antibody was not limiting under these conditions. Background fluorescence due to the second antibody was typically <10% of total fluorescence and was subtracted from total fluorescence to obtain the value for fluorescence due to PAC1 Fab binding. In some experiments, PAC1 Fab binding to platelets was also studied by an enzyme-linked immunosorbent assay in which preactivated platelets were immobilized in microtiter wells before incubation with antibody (11).

PAC1 Fab binding to CHO cells containing recombinant $\alpha_{IIb}\beta_3$ or to low passage endothelial cells containing $\alpha_v\beta_3$ was studied by flow cytometry as described, using the FITC-labeled rabbit anti-mouse Ig as the second antibody (32, 44). Binding studies were carried out with cells resuspended in platelet incubation buffer. In some cases with endothelial cells, attempts were made to "activate" $\alpha_v\beta_3$ with either 0.2 μM phorbol myristate acetate or with an anti-β_3 antibody (anti-LIBS6 Fab) known to induce PAC1 binding to $\alpha_{IIb}\beta_3$ (9). Anti-LIBS6 Fab was provided by Mark Ginsberg (Scripps Research Institute, La Jolla, CA). Binding of the following other monoclonal antibodies to endothelial cells was also studied: A2A9, specific for $\alpha_{IIb}\beta_3$ (14); SSA6, specific for β_3 (14); and LM609, specific for $\alpha_v\beta_3$ (45) (the latter was from David Cheresh, Scripps). The binding of PAC1 IgM to CHO and endothelial cells was measured using FITC-PAC1 IgM (43).

Assay for Measurement of PAC1 Fab Binding to an Activated Form of Purified $\alpha_{IIb}\beta_3$—Activated $\alpha_{IIb}\beta_3$ was purified from a Triton X-100 platelet lysate by RGD-affinity chromatography and immobilized in plastic microtiter wells as described (46). After blocking the wells with bovine serum albumin and washing, PAC1 Fab was added for 4 h at room temperature in the presence of 1 mM $CaCl_2$, 1 mM $MgCl_2$, 150 mM NaCl, and 20 mM Tris-HCl, pH 7.4. After three washes, Fab binding was detected by enzyme-linked immunosorbent assay using a biotinlyated anti-mouse Ig.

Integrin-mediated Tyrosine Phosphorylation—Platelet tyrosine phosphorylation induced by ligand binding to $\alpha_{IIb}\beta_3$ was studied by incubating gel-filtered platelets with fibrinogen (760 nM), PAC1 IgM (50 nM), or PAC1 Fab (1000 nM) for 10 min at 37 °C in the presence or absence of anti-LIBS6 Fab (3 μM) (9). Platelets were then lysed in RIPA buffer, lysates were immunoprecipitated with a polyclonal anti-phosphotyrosine antibody (UP28), and immunoprecipitates were analyzed by immunoblotting for phosphotyrosine-containing proteins using monoclonal anti-phosphotyrosine antibodies (9).

RESULTS

Baculovirus-infected Insect Cells Express and Secrete Antibody PAC1 Fab—In the present studies, we attempted to clone and express a monovalent Fab fragment of PAC1 in the baculovirus system in order to determine the contributions of antibody size, valency, and sequence to the unique integrin-binding properties of this antibody. When Sf9 cells were infected with a recombinant virus containing DNA encoding either the PAC1 γ_1Fd_{His} heavy chain or the PAC1 κ light chain, expression of PAC1 protein within the insect cell cytoplasm could be detected by immunoblotting with antibodies reactive with murine γ_1Fd and κ, respectively. However, the individually expressed heavy and light chains were not secreted by the cells (not shown). In contrast, when insect cells were co-infected with both the PAC1 γ_1Fd_{His} and κ baculoviruses, a single immunoreactive band was now detectable at 50 kDa on nonreduced immunoblots from the

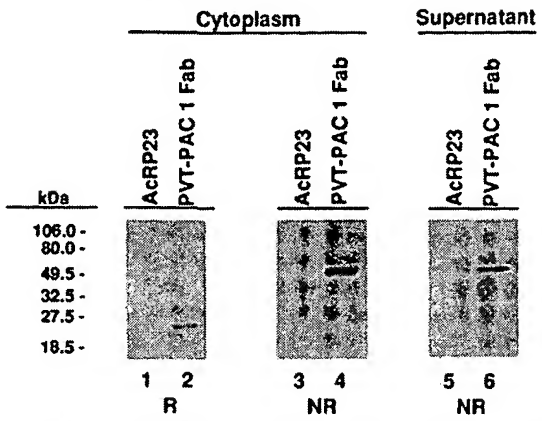

FIG. 2. **Expression of PAC1 Fab in the cytoplasm and cell culture supernatant of Sf9 cells.** Recombinant baculoviruses containing PAC1 γ_1Fd_{His} or PAC1 κ were used to co-infect Sf9 cells for 72 h. Cells infected with the wild-type baculovirus, AcRP23 LacZ, served as a negative control. Sf9 cell cytoplasm and cell culture supernatant were obtained as described under "Materials and Methods" and 3 μg of each were applied to lanes of reduced (*R*) or nonreduced (*NR*) 10% SDS-polyacrylamide gels. Proteins were transferred to nitrocellulose and immunoblotting was carried out using a mixture of two antibodies, one of which (Cg2–236) reacts with PAC1 γ_1Fd and the other (rabbit anti-mouse Ig) with PAC1 κ. Preliminary studies using each detecting antibody alone demonstrated that the upper band at 29 kDa in *lane 2* of the reduced gel represents PAC1 γ_1Fd, while the lower band at 25 kDa is PAC1 κ.

Sf9 cell cytoplasm and the cell culture supernatant (Fig. 2). Reduction of the 50-kDa band by treatment with dithiothreitol generated two bands, one at 29 kDa representing the γ_1Fd_{His} chain and another at 25 kDa representing the κ chain (Fig. 2). Secretion of Fab from these cells was maximal at 72 h.

A Coomassie-stained SDS gel of proteins present in the culture supernatant of co-infected cells showed several bands, including a major band representing a secreted insect cell protein at approximately 60 kDa and a relatively minor band at 50 kDa (Fig. 3, *lane 1*). When the supernatant was passed over a Ni-NTA column, the band at 50 kDa was retained on the column and not detected in the flow-through (Fig. 3*A*, *lane 2*). However, this band represented >90% of the protein that eluted specifically from the column in the presence of 250 mM imidazole (Fig. 3*A*, *lane 4*). After reduction of the eluate with dithiothreitol, two equally prominent Coomassie-stained bands were apparent at 29 and 25 kDa (Fig. 3*A*, *lane 8*). Since the reduced PAC1 γ_1Fd_{His} and κ chains exhibited distinct mobilities, the presence of a single immunoreactive band at 50 kDa under nonreducing conditions suggests that the insect cells were secreting γ_1Fd_{His}-κ heterodimers rather than homodimers. Each 500 ml of cell culture supernatant yielded 5–10 mg of purified PAC1 Fab. The relative migration of PAC1 Fab on a Sephadex G-200 column indicated that it was monomeric and, therefore, monovalent in aqueous solution. Collectively, these experiments show that baculovirus-infected insect cells can secrete substantial amounts of monovalent PAC1 Fab, which can be purified in a single step by immobilized metal chelate affinity chromatography.

PAC1 Fab Is Specific for Integrin $\alpha_{IIb}\beta_3$ on Activated Platelets—The binding of purified PAC1 Fab to platelets was analyzed by flow cytometry using a FITC-labeled polyclonal antibody specific for mouse Ig. No binding of PAC1 Fab was observed when resting platelets were incubated with as much as 1200 nM Fab for 30 min at room temperature. In contrast, PAC1 Fab appeared to bind saturably to platelets activated with 0.2 mM phorbol myristate acetate. Binding was half-maximal at approximately 300 nM Fab and maximal at 600 nM (Fig. 4). Similar results were obtained at 37 °C. The 300 nM value for

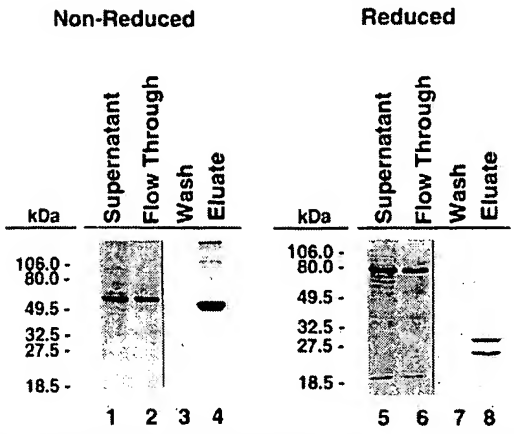

FIG. 3. **Purification of PAC1 Fab secreted from Sf9 cells.** Sf9 cells were co-infected for 72 h with recombinant viruses containing PAC1 γ_1Fd and PAC1 κ. Five-hundred ml of the cell culture supernatant was then dialyzed against phosphate-buffered saline and PAC1 Fab was purified by metal chelate affinity chromatography. Samples from various stages of the purification process were run on nonreduced and reduced 10% SDS-polyacrylamide gels, which were stained for protein with Coomassie Brilliant Blue. *Lanes 1* and *5* represent the dialyzed cell culture supernatant; *lanes 2* and *6*, the flow-through from the Ni-NTA column; *lanes 3* and *7*, samples obtained after extensive washing of the column with 10 mM imidazole; and *lanes 4* and *8*, proteins eluted specifically from the column with 250 mM imidazole. All lanes except *3* and *7* were loaded with 15 μg of protein; *lanes 3* and *7* were loaded with 75 μl of the wash buffer.

half-maximal binding of PAC1 Fab is 60-fold higher than the apparent K_d for PAC1 IgM binding to activated platelets (11). Numerous attempts to label PAC1 Fab with FITC, biotin, or ^{125}I (lactoperoxidase or Bolton-Hunter reagent) resulted in a loss of functional activity, precluding determination of binding constants using directly labeled Fab.

Binding of fibrinogen or PAC1 IgM to platelets is stimulated by physiological agonists, dependent on divalent cations, and inhibited by specific peptides derived from fibrinogen (*e.g.* RGDS or $\gamma_{397-411}$) (13, 47–49). This same pattern of binding was also observed in the case of PAC1 Fab. Fab binding to platelets was stimulated by agonists such as epinephrine, ADP, or thrombin, with half-maximal binding observed at approximately 0.5 μM epinephrine or ADP or 0.01 unit/ml thrombin. Binding of 600 nM PAC1 Fab to activated platelets was inhibited >85% by 10 mM EDTA, 1 mM RGDS, or 1 mM $\gamma_{397-411}$, but not at all by 1 mM RGES. In addition, PAC1 Fab binding was inhibited >85% by a 20-fold molar excess of fibrinogen but not by bovine serum albumin. Conversely, PAC1 Fab inhibited fibrinogen binding to activated platelets in a dose-dependent manner (not shown). For example, 1200 nM PAC1 Fab inhibited the binding of 240 nM fibrinogen by 50%. This concentration of PAC1 Fab is approximately 60-fold higher than the IC_{50} for PAC1 IgM inhibition of fibrinogen binding studied under similar conditions (12).

Like $\alpha_{IIb}\beta_3$, the vitronectin receptor ($\alpha_v\beta_3$) can bind fibrinogen, vitronectin, and von Willebrand factor (50). Since the number of vitronectin receptors on platelets (about 250/cell) is much smaller than the number of $\alpha_{IIb}\beta_3$ complexes (about 80,000/cell) (51, 52), platelets cannot be used to determine whether PAC1 can bind to $\alpha_v\beta_3$. Therefore, PAC1 binding to human umbilical vein endothelial cells, which express $\alpha_v\beta_3$ but not $\alpha_{IIb}\beta_3$, was studied by flow cytometry. Surface expression of $\alpha_v\beta_3$ was verified by binding of antibodies LM609, which is specific for the $\alpha_v\beta_3$ complex (45) and SSA6, specific for β_3 (14) (Fig. 5). No specific binding of PAC1 IgM (40 nM) or PAC1 Fab (600 nM) was detected, either before or after short-term stimulation of the cells with 0.2 μM phorbol myristate acetate. Moreover, no FITC-PAC1 IgM binding was observed in the presence anti-LIBS6 Fab, an anti-β_3 monoclonal antibody that stimulates PAC1 and fibrinogen binding to $\alpha_{IIb}\beta_3$ (9) (Fig. 5). Thus, at antibody concentrations where saturable binding to $\alpha_{IIb}\beta_3$ is observed, PAC1 does not bind to $\alpha_v\beta_3$.

Binding of PAC1 Fab to Activated $\alpha_{IIb}\beta_3$ Does Not Require the Platelet Membrane Microenvironment—The microenvironment of the platelet membrane may play a role in regulating the access of certain antibodies and adhesive ligands to their binding sites on $\alpha_{IIb}\beta_3$ (53). To examine this possibility for PAC1 Fab, activated $\alpha_{IIb}\beta_3$ was extracted from platelets, purified on an RGD column, and immobilized onto plastic (54). PAC1 Fab bound to this activated form of $\alpha_{IIb}\beta_3$ in a dose-dependent and RGDS-inhibitable manner (Fig. 6).

To determine whether PAC1 Fab could bind to the activated form of $\alpha_{IIb}\beta_3$ in the context of a cell other than the platelet, antibody binding to CHO cells expressing either a resting or activated form of recombinant human $\alpha_{IIb}\beta_3$ was examined by flow cytometry. Surface expression of $\alpha_{IIb}\beta_3$ by transfected CHO cells was confirmed using an activation-independent anti-$\alpha_{IIb}\beta_3$ antibody (A2A9). The A5 CHO cell line expresses wild-type $\alpha_{IIb}\beta_3$ in a resting conformation and fails to bind fibrinogen (32, 44). These cells also failed to bind PAC1 Fab or PAC1 IgM (Fig. 7). In contrast, the $\alpha_L\Delta$ CHO cell line expresses a chimeric form of $\alpha_{IIb}\beta_3$ that is constitutively "activated" and capable of binding fibrinogen (33). These cells bound both PAC1 Fab and PAC1 IgM in an RGDS-inhibitable manner (Fig. 7). Thus, whether $\alpha_{IIb}\beta_3$ is studied in its natural platelet membrane environment, in its purified form, or in a heterologous expression system, PAC1 Fab is specific for an activated conformation of the receptor.

PAC1 Fab Binding to Activated $\alpha_{IIb}\beta_3$ Is Dependent on the Structure of H-CDR3—Previous competition studies using synthetic peptides have suggested that an RYD sequence within the H-CDR3 of PAC1 (Arg^{100A}-Tyr^{100B}-Asp^{100C}) is essential for antibody binding to platelets, possibly analogous to the RGD integrin recognition sequences in fibrinogen and von Willebrand factor (16). To test this hypothesis directly, Asp^{100C} in PAC1 Fab was mutated to Glu. An analogous substitution within the RGD tract of von Willebrand factor or vitronectin results in loss of ligand binding to $\alpha_{IIb}\beta_3$ on activated platelets (55, 56). The "RYE" PAC1 Fab mutant failed to bind to activated platelets at antibody concentrations up to 1200 nM, the highest concentration tested by flow cytometry (Fig. 8). Similar results were obtained at PAC1 Fab concentrations up to 3200 nM when antibody binding to phorbol ester-stimulated platelets was studied by enzyme-linked immunosorbent assay (not shown). Immunoblotting experiments showed that the anti-mouse Ig used to detect Fab binding to platelets bound just as well to the RYE mutant as to wild-type PAC1 Fab, and that the mobility of the mutant on SDS gels was identical to that of the wild-type protein (not shown). Thus, a single conservative substitution within the RYD tract of PAC1 exerts a major effect on the ability of the antibody to recognize its integrin target.

Soluble PAC1 IgM but Not PAC1 Fab Stimulates Tyrosine Phosphorylation in Platelets—Fibrinogen binding to unstirred platelets stimulates tyrosine phosphorylation of proteins of 50–68 and 140 kDa. This reaction may require oligomerization of $\alpha_{IIb}\beta_3$ complexes by the dimeric fibrinogen molecule (9). Since PAC1 IgM is multivalent and PAC1 Fab monovalent, we asked if binding of either of these ligands to platelets would trigger tyrosine phosphorylation. Fibrinogen or PAC1 binding was induced by the anti-β_3 antibody, anti-LIBS6, and tyrosine phosphorylation of the 140-kDa substrate was detected by probing platelet immunoblots with antiphosphotyrosine antibodies (9). As expected from previous studies, binding of 750 nM fibrinogen caused prominent tyrosine phosphorylation of the 140-kDa pro-

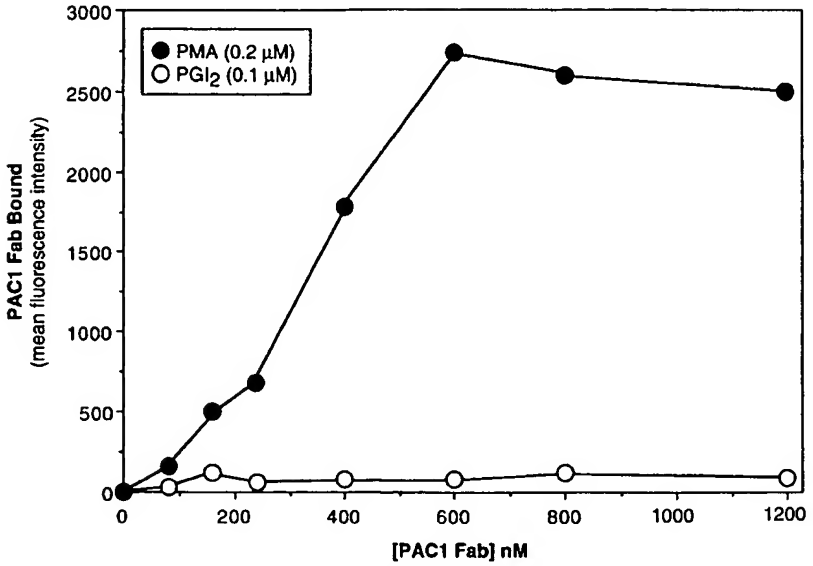

FIG. 4. **Binding of recombinant PAC1 Fab to gel-filtered platelets.** Gel-filtered platelets were incubated at room temperature with the indicated concentrations of PAC1 Fab in the presence of either 0.2 μM phorbol myristate acetate (*PMA*) to maximally stimulate the platelets or 0.1 μM prostaglandin to maintain the platelets in a resting state. After 15 min, a FITC-labeled second antibody reactive with PAC1 Fab was added and the samples incubated an additional 15 min in the dark. Then the amount of PAC1 Fab bound to the platelets was determined by flow cytometry as described under "Materials and Methods." PAC1 binding is expressed as mean fluorescence intensity in arbitrary fluorescence units.

tein (Fig. 9, *lane 3*). Phosphorylation of this protein on tyrosine was also observed in response to a saturating concentration of PAC1 IgM (50 nM) (Fig. 9, *lane 5*). The extent of phosphorylation was much less than that observed with fibrinogen, but it was consistently observed in each of four experiments. In contrast, no tyrosine phosphorylation was observed in response to 1000 nM PAC1 Fab (Fig. 9, *lane 7*). This concentration of PAC1 Fab was sufficient to inhibit the phosphorylation signal induced by PAC1 IgM, indicating that it was binding to $\alpha_{IIb}\beta_3$ under these conditions (not shown). Thus, PAC1 Fab may be unable to induce platelet tyrosine phosphorylation because it is monovalent.

DISCUSSION

Interest in the IgM monoclonal antibody, PAC1, stems from its ability to mimic many of the binding properties of a physiological ligand, fibrinogen, for the platelet integrin $\alpha_{IIb}\beta_3$ (11, 13, 14, 16). The present studies were conducted to begin to formally identify the structural features of PAC1 that are responsible for its unique platelet binding characteristics. Since PAC1 is a large IgM pentamer of approximately 1000 kDa (11), we wanted to establish the relative importance of antibody size, valency, and amino acid sequence as determinants of PAC1 specificity for the activated form of $\alpha_{IIb}\beta_3$. To this end, a recombinant form of PAC1 Fab was cloned and expressed in a baculovirus system and its function was assayed in several different experimental contexts. The results of these studies establish that: 1) the baculovirus system is capable of producing relatively large amounts of monovalent, functional Fab fragments. 2) The size and/or multivalency of PAC1 IgM plays a role in determining antibody affinity but not antibody specificity. 3) The RYD sequence within H-CDR3 is essential for antibody recognition of $\alpha_{IIb}\beta_3$. 4) PAC1 is selective for $\alpha_{IIb}\beta_3$ since it does not recognize the closely related integrin, $\alpha_v\beta_3$, on endothelial cells at antibody concentrations that permit maximal binding to $\alpha_{IIb}\beta_3$. 5) Binding of multivalent PAC1 IgM to $\alpha_{IIb}\beta_3$ stimulates tyrosine phosphorylation in platelets, but binding of monovalent PAC1 Fab does not.

Neither the IgM nor the Fab form of PAC1 bound to unstimulated platelets, whereas both forms appeared to bind saturably

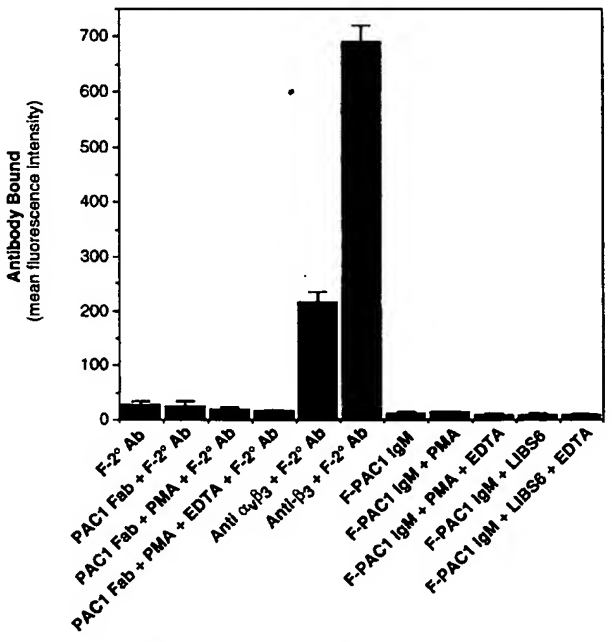

FIG. 5. **PAC1 Fab and PAC1 IgM do not bind to $\alpha_v\beta_3$ on human umbilical vein endothelial cells.** Confluent endothelial cells were detached from the culture dish by incubation with 0.01% trypsin, 0.5 mM EDTA for 3 min, washed once in complete media, and then resuspendend to 2×10^6 cells/ml in platelet incubation buffer. Twenty-μl aliquots were added to tubes containing the indicated antibodies, agonists, or EDTA in a final volume of 50 μl. After a 30-min incubation, 143 μg/ml FITC-labeled rabbit anti-mouse Ig (*F-2° Ab*) was added to all tubes except those containing FITC-PAC1 IgM (*F-PAC1*). After an additional 15 min, samples were diluted and 10,000 cells in each tube were analyzed by flow cytometry. The concentrations of primary antibodies were as follows: PAC1 Fab, 600 nM; anti-$\alpha_v\beta_3$, LM609, 1:100 dilution of ascites; anti-β_3, SSA6, 66 nM; FITC-PAC1 IgM, 40 nM. In certain tubes, either PMA (0.2 μM) or anti-LIBS6 Fab (3 μM) was used in an attempt to activate $\alpha_v\beta_3$. EDTA at 5 mM was present in some tubes to prevent specific PAC1 binding. Data represent the means ± S.D. of triplicate determinations from a single experiment representative of two so performed.

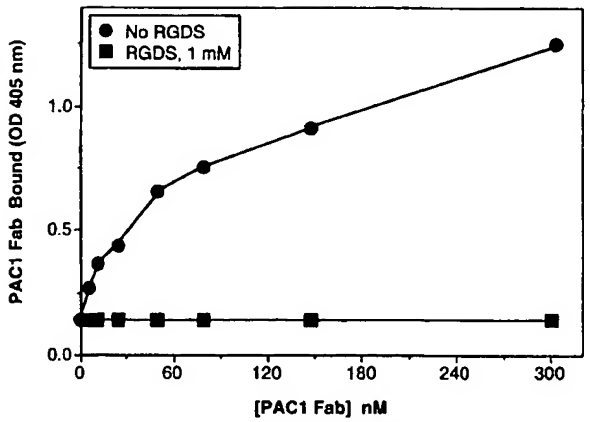

FIG. 6. **Binding of PAC1 Fab to purified, activated $\alpha_{IIb}\beta_3$.** PAC1 Fab was incubated in the presence or absence of 1 mM RGDS for 4 h in microtiter wells coated with an activated form of purified $\alpha_{IIb}\beta_3$. After washing, PAC1 Fab binding was detected by enzyme-linked immunosorbent assay.

to agonist-stimulated platelets (Fig. 4) (11). Like the binding of fibrinogen and von Willebrand factor, binding of PAC1 Fab was inhibited by divalent cation chelation with EDTA and by excess fibrinogen or RGDS, but not by the control peptide, RGES. Recently, small amounts of fibrinogen and von Willebrand factor have been expressed in mammalian cell expression systems, and mutational analyses of these proteins have begun to examine the role of the RGD sequences in binding to $\alpha_{IIb}\beta_3$ (55, 57). Given the similarities in the binding characteristics of PAC1 and these natural ligands, and the crystallographic data base that already exists for antibodies, further structural analysis of PAC1 Fab may provide information relevant to the interaction of the larger adhesive proteins with $\alpha_{IIb}\beta_3$.

Experimental evidence suggests that platelet activation causes changes in $\alpha_{IIb}\beta_3$ that permit access of PAC1 and several RGD-containing adhesive proteins to the receptor (11, 58). The precise nature and magnitude of the structural changes that $\alpha_{IIb}\beta_3$ must undergo is unknown. However, ligand size may represent one determinant of the final interaction. In support of this concept, synthetic RGD peptides or peptidomimetics ($\leq$2 kDa) bind to $\alpha_{IIb}\beta_3$ whether or not platelet activation has taken place (59). The same appears to hold true for larger RGD-containing peptides of 5–10 kDa derived from certain snake venoms or leeches, although in many of these cases platelet activation can increase apparent binding affinity by up to 10-fold (60, 61). Moreover, Coller (62) has shown that the rate of binding of antibody 7E3 to $\alpha_{IIb}\beta_3$ was influenced by the size of the antibody as it was varied from a 50-kDa Fab fragment to a 600-kDa cross-linked tetramer. However, unlike PAC1, even the large 600-kDa tetramer of 7E3 bound to resting platelets (62). The epitope for 7E3 is clearly distinct from that of PAC1 because 7E3 binding is neither divalent cation-dependent nor inhibited by fibrinogen-derived peptides. Therefore, the importance of the size of fibrinogen or PAC1 for their selective binding to activated $\alpha_{IIb}\beta_3$ has remained an open question.

The present studies demonstrate that measurable binding of PAC1 to platelets is dependent on the state of $\alpha_{IIb}\beta_3$ activation, regardless of whether the antibody is a monovalent 50-kDa Fab fragment or a 1000-kDa IgM pentamer. In fact, the microenvironment of the platelet membrane was not even necessary for this binding to activated $\alpha_{IIb}\beta_3$ since PAC1 Fab also bound selectively to an activated form of the receptor expressed in CHO cells (Fig. 7). Thus, the specificity of PAC1 for an active conformation of $\alpha_{IIb}\beta_3$ must be defined by structural features intrinsic to the receptor and to the antibody-combining site of PAC1. In the case of antibody-protein complexes that have been resolved crystallographically, the surface area of the antibody-combining site has ranged between 700 and 900 Å^2 (63, 64). The putative ligand-binding pocket of $\alpha_{IIb}\beta_3$ appears to reside in the amino-terminal globular head of the integrin (58). Rotary-shadowed electron micrographs of $\alpha_{IIb}\beta_3$ indicate that this globular head is 80 × 120 Å, potentially encompassing a surface area much larger than the antibody-combining site of PAC1 (65, 66). At this same level of resolution, the shape of the globular head of $\alpha_{IIb}\beta_3$ is not grossly modified by fibrinogen binding (66, 67). Thus, the intrinsic changes in the receptor that regulate ligand binding are likely to be relatively subtle.

The multimeric or multivalent structure of PAC1 did not affect antibody specificity, but it did influence two other features of the interaction of the antibody with $\alpha_{IIb}\beta_3$, namely the apparent affinity of the reaction and the ability of the antibody to induce outside-in signaling across the receptor. A rough estimate of the differences in apparent affinity between PAC1 Fab and PAC1 IgM can be obtained by comparing the dose-dependence of their binding to activated platelets as well as their relative potencies in inhibiting fibrinogen binding to platelets. Half-maximal binding of PAC1 Fab to activated platelets was observed at approximately 300 nM (Fig. 4), a value 60-fold higher than the apparent K_d for PAC1 binding (11). Similarly, PAC1 Fab was approximately 60-fold less potent than PAC1 IgM in causing half-maximal inhibition of fibrinogen binding to platelets. These differences may relate to the differences in valency between the two forms of the antibody.

Binding of the dimeric fibrinogen molecule to platelets stimulates tyrosine phosphorylation of protein substrates of 50–68 and 140 kDa, while binding of monomeric plasmin-derived fragments of fibrinogen does not (9). Similarly, binding of PAC1 IgM to platelets consistently induced a weak tyrosine phosphorylation response, but PAC1 Fab did not (Fig. 9). In contrast to these results with the soluble Fab, tyrosine phosphorylation is observed when platelets become adherent to PAC1 Fab that is immobilized on a plastic surface.[2] The pattern of tyrosine phosphorylation is the same as that observed during platelet adhesion to fibrinogen (10). Taken together, these observations suggest that cross-linking of $\alpha_{IIb}\beta_3$ complexes within the plane of the plasma membrane may be required for $\alpha_{IIb}\beta_3$-initiated outside-in signaling in platelets. The much stronger tyrosine phosphorylation response to fibrinogen than to PAC1 IgM in Fig. 9 may be due to a more favorable geometry in the case of the natural ligand for cross-linking adjacent receptors. Alternatively, it may be due to fundamental differences between the physiological ligand and the antibody with respect to their interactions with the receptor.

A single point mutation in the H-CDR3 of PAC1 in which Asp^{100C} was converted to Glu resulted in a loss of antibody binding to $\alpha_{IIb}\beta_3$, at least at antibody concentrations up to 3200 nM. This mutation converts the core RYD sequence in PAC1 to RYE, strongly suggesting that the RYD tract within this particular CDR is an essential component of the antibody-combining site of PAC1. These results are consistent with molecular modeling studies which predicted that the RYD backbone of PAC1 is oriented at the apex of a β-loop formed by this CDR (17). This would be similar to the backbone orientation of several bioactive RGD peptides and disintegrins that have been subjected to high resolution structural analyses (19–21). In particular, the relative spatial orientation of the Arg-NH_3^+ and Asp-CO_2^- groups in the RGD region of bioactive peptides appears essential for integrin recognition (20). The same may hold true for the Arg and Asp in the RYD tract of PAC1, ex-

[2] S. J. Shattil, M. Cunningham, J. Deng, and C. Abrams, unpublished observations.

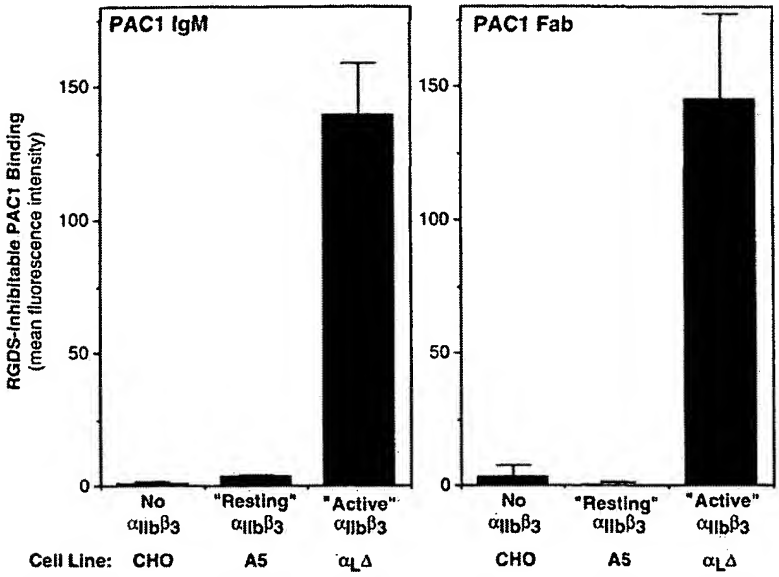

FIG. 7. **PAC1 IgM and PAC1 Fab bind specifically to an activated form of recombinant $\alpha_{IIb}\beta_3$ expressed in CHO cells.** Mock-transfected CHO cells, A5 cells expressing the wild-type, "resting" form of $\alpha_{IIb}\beta_3$, and $\alpha_L\Delta$ cells expressing an activated form $\alpha_{IIb}\beta_3$ were suspended to 4×10^6 cells/ml in platelet incubation buffer. PAC1 IgM (7 nM) or PAC1 Fab (428 nM) was added for 30 min. Then FITC-labeled rabbit anti-mouse Ig (143 μg/ml) was added for 15 min and PAC1 binding was quantitated by flow cytometry. Specific PAC1 binding is expressed in arbitrary fluorescence units as "RGDS-inhibitable" binding. This was defined as binding that was inhibited by 1 mM RGDS, and it represented >90% of total binding. Data represent means ± S.D. of triplicate determinations of a single experiment representative of two so performed.

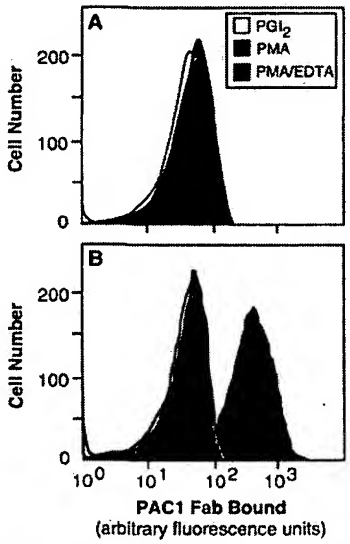

FIG. 8. **The RYE mutant of PAC1 Fab fails to bind to activated platelets.** Wild-type PAC1 Fab contains an Arg-Tyr-Asp (RYD) sequence at residues 100A-C of the CDR3 heavy chain. The RYE mutant contains a Glu at residue 100C instead of Asp. 1200 nM RYE PAC1 Fab mutant (*panel A*) or wild-type PAC1 Fab (*panel B*) were incubated for 15 min with either resting platelets (PGI_2, 0.1 μM), platelets stimulated with 0.2 μM PMA, or PMA-stimulated platelets in the presence of 5 mM EDTA. After addition of FITC-labeled anti-mouse Ig, PAC1 Fab binding was determined by flow cytometry. In these histograms, PAC1 Fab binding is expressed in arbitrary fluorescence units on a log scale. This experiment is representative of three so performed. PGI_2, prostaglandin I_2.

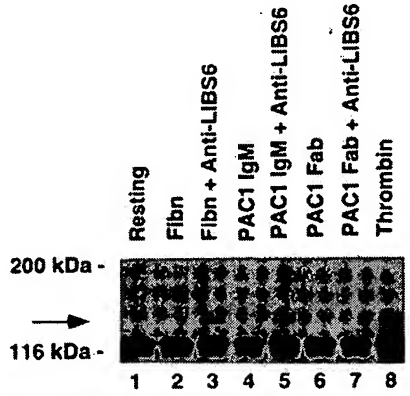

FIG. 9. **Effect of PAC1 binding on platelet tyrosine phosphorylation.** Platelets were incubated for 10 min with 760 nM fibrinogen, 50 nM PAC1 IgM, or 1000 nM PAC1 Fab. In some tubes, the anti-β_3 antibody Fab, anti-LIBS6 (3 μM), was present to promote binding of each of these ligands to $\alpha_{IIb}\beta_3$. Platelets were then processed as described under "Materials and Methods" to assess tyrosine phosphorylation of a 140-kDa platelet protein (*arrow*), which was previously shown to become phosphorylated in response to fibrinogen binding (9). *Lane 1* is a negative control and represents unstimulated platelets in the absence of added fibrinogen. Not shown is the fact that anti-LIBS6 in the absence of fibrinogen also failed to stimulate tyrosine phosphorylation. *Lanes 3* and *8* are positive controls showing the effect of fibrinogen binding in response to either anti-LIBS6 (*lane 3*) or 0.5 unit/ml thrombin (*lane 8*). Note the positive but weak response to PAC1 IgM binding (*lane 5*) and the lack of a response to PAC1 Fab (*lane 7*).

plaining the inability of the RYE mutant to recognize $\alpha_{IIb}\beta_3$.

Recently, filamentous phage have been engineered to express an anti-HIV antibody in which the RGD sequence was inserted into the H-CDR3 region (68). These phage bound equally well to $\alpha_{IIb}\beta_3$ and $\alpha_v\beta_3$. In contrast, we found that PAC1 failed to bind to $\alpha_v\beta_3$ on human umbilical vein endothelial cells at antibody concentrations that appeared to be saturable with respect to $\alpha_{IIb}\beta_3$ (Fig. 5). Since it is not known whether the affinity of $\alpha_v\beta_3$ is modulated by inside-out signaling in these cells, attempts were made to activate either the cells with phorbol myristate acetate or the receptor directly with an anti-β_3 antibody (anti-LIBS6). Neither of these reagents stimulated PAC1 binding. The exact degree of selectivity of PAC1 for $\alpha_{IIb}\beta_3$ over $\alpha_V\beta_3$ will require additional study. However, such selectivity may relate to the core RYD sequence, to specific H-CDR3 residues that flank RYD, and/or to other CDRs that contribute to the antibody-combining site. Of interest in this regard, the disintegrin, barbourin, contains a lysine instead of an arginine in a core KGD sequence, and this confers $\alpha_{IIb}\beta_3$ selectivity to the mol-

ecule (23, 25). It can be anticipated that genetically engineered antibodies, including PAC1, will be useful in identifying some of the factors responsible for the affinity and specificity of RGD-containing ligands for integrin adhesion receptors (69).

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